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Effect of fatty acids on hyphal growth in the pathogenic yeast *Candida albicans*

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Cette thèse intitulée :

Effect of fatty acids on hyphal growth in the pathogenic yeast *Candida albicans*

Présentée par :
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Résumé

Candida albicans est une levure pathogène qui, à l'état commensal, colonise les muqueuses de la cavité orale et du tractus gastro-intestinal. De nature opportuniste, *C. albicans* cause de nombreuses infections, allant des candidoses superficielles (muguet buccal, vulvo-vaginite) aux candidoses systémiques sévères. *C. albicans* a la capacité de se développer sous diverses morphologies, telles que les formes levures, pseudohyphes et hyphes. Des stimuli environnementaux mimant les conditions retrouvées chez l'hôte (température de 37°C, pH neutre, présence de sérum) induisent la transition levure-à-hyphe (i.e. morphogenèse ou filamentation). Cette transition morphologique contribue à la pathogénicité de *C. albicans*, du fait que des souches présentant un défaut de filamentation sont avirulentes. Non seulement la morphogenèse est un facteur de virulence, mais elle constituerait aussi une cible pour le développement d'antifongiques. En effet, il a déjà été démontré que l'inhibition de la transition levure-à-hyphe atténuait la virulence de *C. albicans* lors d'infections systémiques. Par ailleurs, des études ont démontré que de nombreuses molécules pouvaient moduler la morphogenèse. Parmi ces molécules, certains acides gras, dont l'acide linoléique conjugué (CLA), inhibent la formation d'hyphes. Ainsi, le CLA posséderait des propriétés thérapeutiques, du fait qu'il interfère avec un déterminant de pathogénicité de *C. albicans*. Par contre, avant d'évaluer son potentiel thérapeutique dans un contexte clinique, il est essentiel d'étudier son mode d'action.

Ce projet vise à caractériser l'activité anti-filamentation des acides gras et du CLA et à déterminer le mécanisme par lequel ces molécules inhibent la morphogenèse chez *C. albicans*. Des analyses transcriptomiques globales ont été effectuées afin d'obtenir le profil transcriptionnel de la réponse de *C. albicans* au CLA. L'acide gras a entraîné une baisse des niveaux d'expression de gènes encodant des protéines hyphes-spécifiques et des régulateurs de morphogenèse, dont *RAS1*. Ce gène code pour la GTPase Ras1p, une protéine membranaire de signalisation qui joue un rôle important dans la transition levure-à-hyphe. Des analyses de PCR quantitatif ont confirmé que le CLA inhibait l'induction de

RAS1. De plus, le CLA a non seulement causé une baisse des niveaux cellulaires de Ras1p, mais a aussi entraîné sa délocalisation de la membrane plasmique. En affectant les niveaux et la localisation cellulaire de Ras1p, le CLA nuit à l'activation de la voie de signalisation Ras1p-dépendante, inhibant ainsi la morphogenèse. Il est possible que le CLA altère la structure de la membrane plasmique et affecte indirectement la localisation membranaire de Ras1p. Ces travaux ont permis de mettre en évidence le mode d'action du CLA. Le potentiel thérapeutique du CLA pourrait maintenant être évalué dans un contexte d'infection, permettant ainsi de vérifier qu'une telle approche constitue véritablement une stratégie pour le traitement des candidoses.

Mots clés: *Candida albicans*, morphogenèse, transition levure-à-hyphe, acides gras, acide conjugué linoléique, Ras1p, voie de signalisation

Summary

The yeast *Candida albicans* is an inhabitant of the oral cavity, the gastrointestinal and genitourinary tracts of humans. Generally encountered as a commensal, it is also an opportunistic pathogen that causes a spectrum of infections, ranging from superficial mycoses (thrush, vulvovaginitis) to severe and life-threatening systemic infections. A striking feature of *C. albicans* is its ability to grow in different morphological forms, including budding yeasts, pseudohyphae, and hyphae. Environmental cues that mimic host conditions (elevated temperature, neutral or alkaline pH, and serum) induce the yeast-to-hypha transition. Morphogenesis is considered to be an attribute of pathogenesis, as mutants locked as yeasts or filamentous forms are avirulent. Given that the yeast-to-hypha transition is a virulence factor, it may also constitute a target for the development of antifungal drugs. Indeed, evidence has shown that impairing morphogenesis is a means to treat systemic candidiasis. Concurrently, a number of molecules have been reported to modulate morphogenesis in *C. albicans*. For instance, several fatty acids, including conjugated linoleic acid (CLA), inhibited the yeast-to-hypha transition. By interfering with an important attribute of *C. albicans* pathogenesis, CLA may harbor antifungal properties. However, before assessing its therapeutic potential in a clinical context, it is mandatory to address CLA's mode of action.

The present study aims to further characterize the hypha-inhibiting properties of fatty acids and CLA and to elucidate the mechanism by which these molecules inhibit the yeast-to-hypha transition in *C. albicans*. Gene expression analyses were performed to gain insight into the transcriptional response of cells to CLA on a genome-wide scale and to probe the fatty acid's mode of action. CLA downregulated the expression of hypha-specific genes and blocked the induction of genes encoding regulators of hyphal growth, including that of *RAS1*, which encodes the small GTPase Ras1p. A membrane-associated signaling protein, Ras1p plays a major role in morphogenesis. Quantitative PCR analyses showed that CLA prevented the increase in *RAS1* mRNA levels which occurred at the onset of the

yeast-to-hypha transition. Unexpectedly, CLA reduced the steady-state levels of Ras1p. Additionally, CLA caused the delocalization of GFP-Ras1p from the plasma membrane. These findings indicate that CLA treatment results in suboptimal Ras1p cellular concentrations and localization, which impedes Ras1p signaling and inhibits the yeast-to-hypha transition. CLA may indirectly affect Ras1p localization by altering the structure of the plasma membrane. These studies have provided the mechanism underlying CLA's hypha-inhibiting properties and may serve as the rationale to examine CLA's therapeutic potential in the context of a *Candida* infection. There is a general lack of clinical evidence demonstrating that impairing morphogenesis is a sound approach to treat candidiasis. To remedy this situation, the therapeutic potential of molecules that modulate morphogenesis, such as CLA, should be clinically assessed.

Keywords: *Candida albicans*, yeast-to-hypha transition, morphogenesis, hyphal growth, fatty acids, conjugated linoleic acid, Ras1p signaling

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À mes parents, parce qu'ils m'ont beaucoup appris, un peu à leur insu.

"All we have is now"
The Flaming Lips, 2002

List of abbreviations

3OC12HSL: 3-oxo-C12-acyl homoserine lactone
5-FC: 5-flucytosine
5-FOA: 5-fluoroorotic acid
5-FU: 5-fluorouracil
A: adenine
AIDS: Acquired immune deficiency syndrome
AMP: adenosine monophosphate
ATP: adenosine triphosphate
BEC: buccal epithelial cells
bHLH: basic helix-loop-helix
bp: base pairs
C.: *Candida*
C: cytosine
cAMP: cyclic adenosine monophosphate
CDK: cyclin-dependent kinase
cDNA: complementary DNA
CLA: conjugated linoleic acid
COX: cyclooxygenase
CRIB: Cdc42/Rac interactive binding
CSP: competence-stimulating peptide
C_T: threshold cycle
DAB: 1,4-diamino-2-butanone
DAPI: 4'-6'diamidino-2-phenyl-indole
DHA: docosahexaenoic acid
DIC: differential interference contrast
DNA: deoxyribonucleic acid
DOC: deoxycholate
Dox: doxycycline
EBI: ergosterol biosynthesis inhibitors
EC: esophageal candidiasis
ESCRT: endosomal-sorting complex required for trafficking
FBS: fetal bovine serum
FLP: flippase
FRT: flippase recognition target
FTS: S-farnesylthiosalicylic acid
G: guanine
GAP: GTPase-activating protein
GDP: guanosine diphosphate
GEF: guanine nucleotide exchange factor
GFP: green fluorescent protein

GI: gastrointestinal
GlcNAc: N-acetylglucosamine
GO: gene ontology
GPI: glycosylphosphatidylinositol
GRACE: gene replacement and conditional expression
GTP: guanosine triphosphate
HA: hemagglutinin
HDAC: histone deacetylase
HIV: human immunodeficiency virus
HSG: hypha-specific gene
HUVEC: human umbilical vein endothelial cells
IL: interleukin
kDa: kilodalton
MAP: mitogen-activated protein
MIC: minimal inhibitory concentration
mRNA: messenger RNA
MTL: mating-type-like
NRE: Nrg1p response element
OD: optical density
OPC: oropharyngeal candidiasis
PAGE: polyacrylamide gel electrophoresis
PAK: p21-activated kinase
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PKA: protein kinase A
PKC: protein kinase C
RA: Ras-association
rDNA: ribosomal deoxynucleic acid
RIPA: radioimmunoprecipitation assay
RNA: ribonucleic acid
S.: *Saccharomyces*
SAHA: suberoylanilide hydroxamic acid
SAP: secreted aspartic protease
SDS: sodium dodecyl sulfate
SEM: scanning electron microscopy
SLAD: synthetic low ammonium dextrose
T: thymine
TBS-T: tris-buffered saline Tween 20
Tor: target of rapamycin
tRNA: transfer RNA
TSA: trichostatin A
TX: thromboxane
UFA: unsaturated fatty acid

VVC: vulvovaginal candidiasis
YNB: yeast nitrogen base
YPD: yeast peptone dextrose
μM: micromolar
μm: microns

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Introduction

The growing population of immunocompromised humans has allowed many fungi to become pathogens. The most common agents of fungal infections include *Candida*, *Aspergillus*, and *Cryptococcus* (Pappas, 2010). In the genus *Candida*, almost 200 species of ascomycetous, asexual yeasts are currently listed, yet only *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *C. lusitaniae* are encountered as opportunistic pathogens of humans (Odds *et al.*, 2007). *C. albicans* is the most common cause of invasive fungal infections in hospital settings, in addition to being the causative agent of superficial mycoses such as oral thrush and vulvovaginitis (Horn *et al.*, 2009; Miceli *et al.*, 2011) (discussed in section 1.5).

The first description of (oral) thrush, an infection of the mucous membranes of the mouth and throat, dates back to 400 B. C. and was made by Hippocrates who mistook the infection for oral ulcers (Adams, 1939). It was not until 1846 that thrush was shown to be caused by a contagion rather than by abnormalities of the host (Berg, 1846). Using a "scientific approach", Berg reproduced thrush in healthy newborns by infecting them with material from aphthous lesions. While Berg's experiments demonstrated that thrush was caused by an organism (i.e. a fungus), it was not identified correctly. Confusion regarding the true identity of the fungus was perpetuated until 1923, when Berkhout proposed that the organism causing thrush should bear the generic name *Candida* (Berkhout, 1923). In 1954, the nomen conservandum (*C. albicans*) was officially approved (Calderone, 2002). The name *Candida* is derived from the Latin phrase "toga candida" which was used to describe the special white robe worn by candidates for the Roman Senate; *albicans* means "to whiten". The name "*Candida albicans*" may refer to the oral lesions of aphthae or thrush or to the characteristic white colonies produced by *C. albicans* on agar (Calderone, 2002).

C. albicans is now ranked as the third or fourth most common agent of microbial septicemia in hospitals, having surpassed many bacterial infections in terms of incidence and morbidity (Almirante *et al.*, 2005; Beck-Sague & Jarvis, 1993; Tortorano *et al.*, 2004).

These severe, often lethal *Candida* infections are on the rise due to a growing population of hospitalized patients with underlying immune deficiencies stemming from treatment for cancer or immunosuppression following a transplantation (Enoch *et al.*, 2006). Treatment of such infections is complicated by the limited arsenal of antifungal drugs, the severe side effects in patients, the development of antifungal drug resistance, and the emergence of species refractory to conventionally used agents (Sanglard & Odds, 2002). There is a need for new targets or new strategies in antifungal therapy (discussed in section 1.6).

An Ascomycete, *C. albicans* belongs to the Saccharomycotina subphylum, to the Hemiascomycetes class, and to the *Candida albicans* clade (Figure 1.1) (Taylor, 2007). Baker's yeast *Saccharomyces cerevisiae* also belongs to the Hemiascomycetes class, which makes it a close relative of *C. albicans*. Both yeasts diverged approximately 200 million to 800 million years ago (Hedges, 2002). Yet, about two-thirds of the ~6,500 genes in the *C. albicans* genome have clear homologues in *S. cerevisiae*, allowing many gene functions to be inferred and key aspects of various processes in the pathogenic to be understood (discussed in 1.3). Additionally, both yeasts are similar in their cellular morphologies, their ability to metabolize fermentable carbon sources, and their capacity to reproduce asexually by budding. A parasexual cycle has been identified in *C. albicans*, but differs from the complete sexual cycle described for *S. cerevisiae* because the pathogenic yeast is diploid, does not exist naturally in a haploid form, and does not undergo meiosis (discussed in 1.1.3).

A striking feature that differentiates *C. albicans* from *S. cerevisiae* and other *Candida* species is its ability to grow in a variety of morphological forms, including as budding yeasts, as pseudohyphae, and as true hyphae (Sudbery *et al.*, 2004) (discussed in section 1.1). The yeast-to-hypha transition, also known as morphogenesis or filamentation, is induced by a variety of environmental cues (discussed in section 1.2) which activate a complex network of signaling pathways composed of transducers, kinases, and transcription factors (Biswas *et al.*, 2007; Brown *et al.*, 2007; Ernst, 2000) (discussed in

section 1.3). These signaling pathways are involved in sensing and transmitting inducing signals and in regulating the regulatory and structural elements essential for hyphal growth. Interestingly, the same pathways regulate the expression of virulence factors of *C. albicans* such as cell surface adhesins, secreted aspartic proteases, lipases, and phospholipases (discussed in section 1.4). Because it contributes to the overall pathogenesis of *C. albicans*, morphogenesis also constitutes one of its virulence factors (see section 1.4.3).

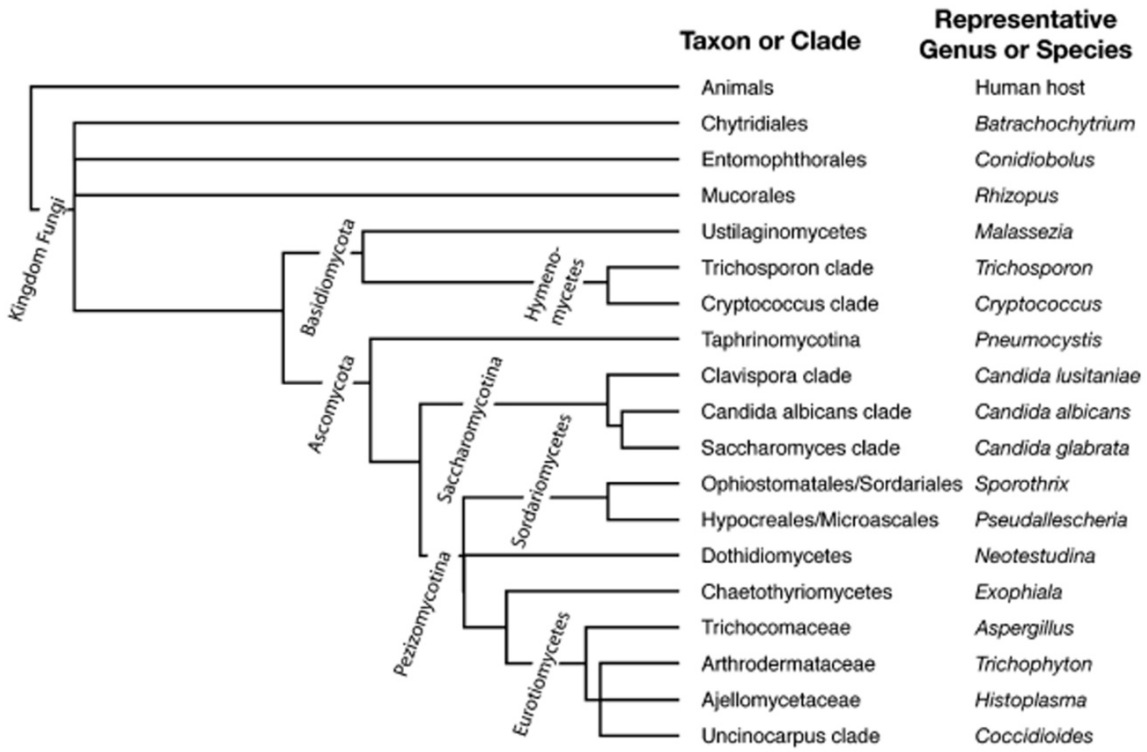

Figure 1. 1 Phylogenetic tree of fungi pathogenic to humans and animals.

A maximum-likelihood phylogeny based on small-subunit rDNA sequences of diverse fungi. Taxa of commonly encountered pathogenic fungi were added to the phylogeny. Adapted from Taylor (2007).

It has been suggested that virulence factors of *C. albicans* may be potential targets for the development of new antifungal drugs (see section 1.6.2). One such virulence factor that could be targeted by antifungal agents is morphogenesis. Interestingly, many small molecules have been reported to modulate the yeast-to-hypha transition. For instance, fatty acids, including conjugated linoleic acid (CLA), inhibited hyphal growth in *C. albicans* (Clement *et al.*, 2007). Thus, CLA and fatty acids may be candidates for the development of novel antifungal agents. However, understanding how fatty acids inhibit morphogenesis is a prerequisite before undertaking studies to assess their therapeutic potential. In the present study, CLA is used to probe the mechanism by which fatty acids inhibit hyphal growth in *C. albicans*.

This thesis is divided into four major sections. In Chapter 1, morphogenesis in *C. albicans* is described in depth, in terms of the different morphological growth forms, inducing signals, and molecular events involved in the morphogenetic transition. The association between the yeast-to-hypha transition and virulence is described, demonstrating why morphogenesis may constitute a target for the development of antifungal drugs. In Chapter 2, findings pertaining to the hypha-inhibiting effects of CLA and to its mode of action are presented. These results were published in an article entitled "Conjugated linoleic acid inhibits hyphal growth in *Candida albicans* by modulating Ras1p cellular levels and downregulating *TEC1* expression" in the scientific journal Eukaryotic Cell. In Chapter 3, a review on small molecules that modulate morphogenesis is presented. This minireview entitled "Modulation of morphogenesis in *Candida albicans* by various small molecules" was submitted to the scientific journal Eukaryotic Cell and is presently in revision. Finally, in Chapter 4, issues that are relevant to this work are discussed and perspectives are proposed.

1. Chapter 1. Literature review

In this chapter, the various aspects of morphogenesis, including cell morphologies, morphogenetic signals and signaling pathways, and downstream targets of these signaling pathways, are reviewed. The different types of *Candida* infections are described. Conventional antifungal drugs available to treat such infections as well as novel treatments are also discussed. Most of the notions constituting the backdrop of this study are provided in this chapter.

1.1 Cell morphologies of *C. albicans*

C. albicans was initially considered to be dimorphic, as it can grow in a budding yeast form or as branching, filamentous forms such as pseudohyphae and true hyphae. However, the pathogenic yeast also exists in a mating-competent opaque form (Bennett & Johnson, 2005; Slutsky *et al.*, 1987) and can be induced to form thick-walled spherical cells known as chlamydospores (Staib & Morschhauser, 2007). The term pleiomorphic appears to be better suited to describe *C. albicans*, as it fully reflects the spectrum of its morphologies (Figure 1.2).

1.1.1 Pseudohyphae and true hyphae

Even though pseudohyphae superficially resemble hyphae, both morphological forms have different cell shapes. A feature that characterizes hyphal cells is the absence of constrictions at the mother-daughter junction or at subsequent septal junctions, which results in cells with parallel sides along their entire length (Figure 1.2C). In contrast, pseudohyphae have constrictions at the mother-daughter junction and between each individual cellular compartment (Sudbery *et al*., 2004) (Figure 1.2B). Sides of pseudohyphal cells are not parallel, resulting in compartments being wider in the middle than at the two ends (Merson-Davies & Odds, 1989). Additionally, hyphae are narrower than pseudohyphae, with a width of ~2 μm compared to ~2.8 μm-5 μm, respectively (Sevilla & Odds, 1986).

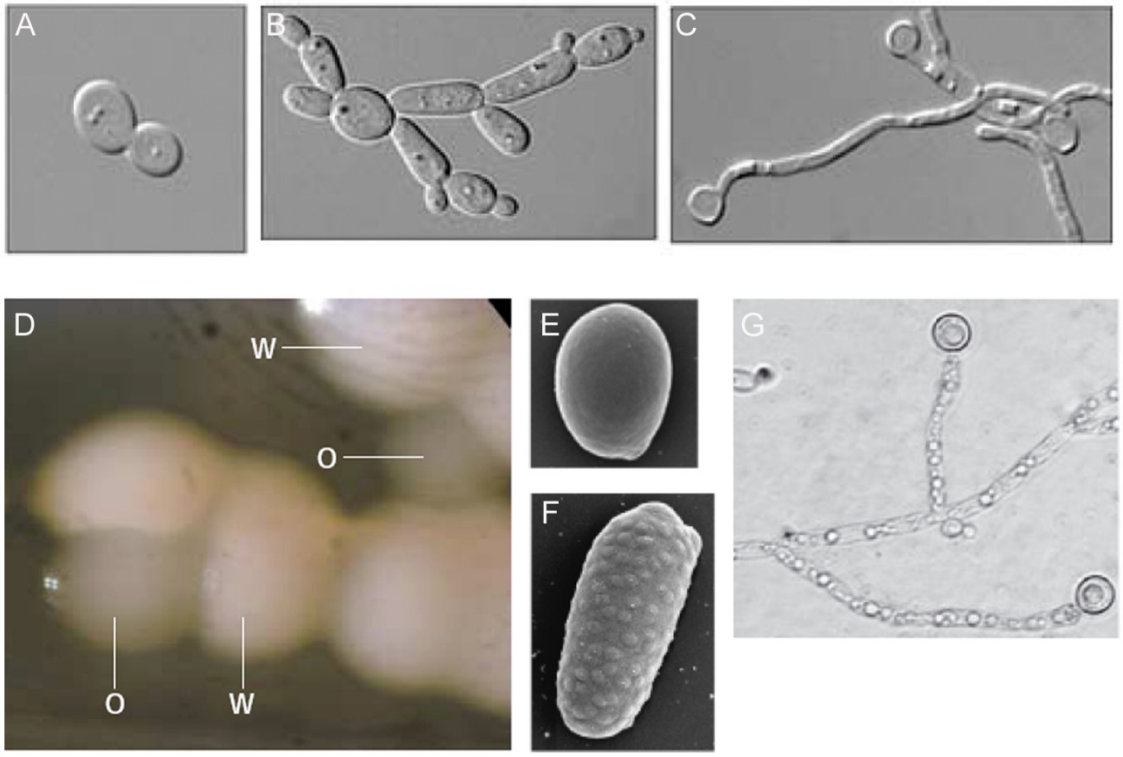

Figure 1. 2 Morphological forms of *Candida albicans*.

Yeasts (A), pseudohyphae (B), and hyphae (C). Budding yeast cells are similar to diploid *S. cerevisiae* cells. Pseudohyphal cells have constrictions at the mother-daughter junction and at the positions of septa. Hyphae have parallel cell walls and no constrictions. (D) White-opaque phenotypic switching of the *C. albicans* WO-1 strain, grown on salt-dextrose at 23°C for three days. White (W) and opaque (O) colonies are seen. The cellular phenotype of white (E) and opaque cells (F). The white cell is round with a relatively smooth surface while the opaque cell is twice the size of the white cell and has unique wall pimples. (G) Chlamydospores are thick-walled spherical cells that are ~3 to 4 times larger than normal yeast cells. Adapted from Brown *et al*. (2007); Berman & Sudbery (2002); Staib & Morschhauser (2007).

C. albicans undergoes reversible morphological transitions between unicellular budding yeast cells and filamentous growth forms such as pseudohyphae and true hyphae. A variety of environmental conditions induce the switch from yeast growth to filamentous growth (Sudbery *et al.*, 2004) (see section 1.2). Pseudohyphae develop into elongated buds that remain attached to the mother cells, resulting in filaments of elongated cells with constrictions at the septa. In contrast, true hyphae grow by continuous apical extension followed by septation. Although morphological and cell cycle studies suggest that pseudohyphal and hyphal growth forms represent distinct developmental stages (Berman & Sudbery, 2002; Sudbery *et al.*, 2004), recent work has demonstrated that the pseudohyphal morphology is not an alternate fate, but rather a true intermediate state between the yeast and hyphal morphologies (Carlisle *et al.*, 2009).

Several aspects of the cell cycle of hyphae and pseudohyphae also differ (Figure 1.3). In pseudohyphal cells, the daughter bud emerges from the mother cell at the start of the cell cycle. In hyphae, germ tube formation occurs before the start of the cell cycle, and is thus cell cycle-independent (Hazan *et al.*, 2002). Polarized growth in yeast and pseudohyphal cells involves the polarisome, while apical extension of the germ tube is driven by the Spitzenkörper. Specific to hyphal cells, this membranous structure is usually found in strictly filamentous fungi and resembles the yeast polarisome (Whiteway & Bachewich, 2007). In pseudohyphal cells, the first septum is laid down at the mother-daughter junction, whereas in hyphae, it is formed within the growing germ tube (Sudbery *et al*., 2004). The position of the septum affects the position of the first mitosis, which occurs at the mother-daughter junction in pseudohyphae, but entirely within the germ tube in hyphae. The absence of cytokinesis is common to both pseudohyphal and hyphal growth modes, which results in cells remaining attached. However, while hyphae appear as long, septated filaments, pseudohyphae have constrictions at the septa.

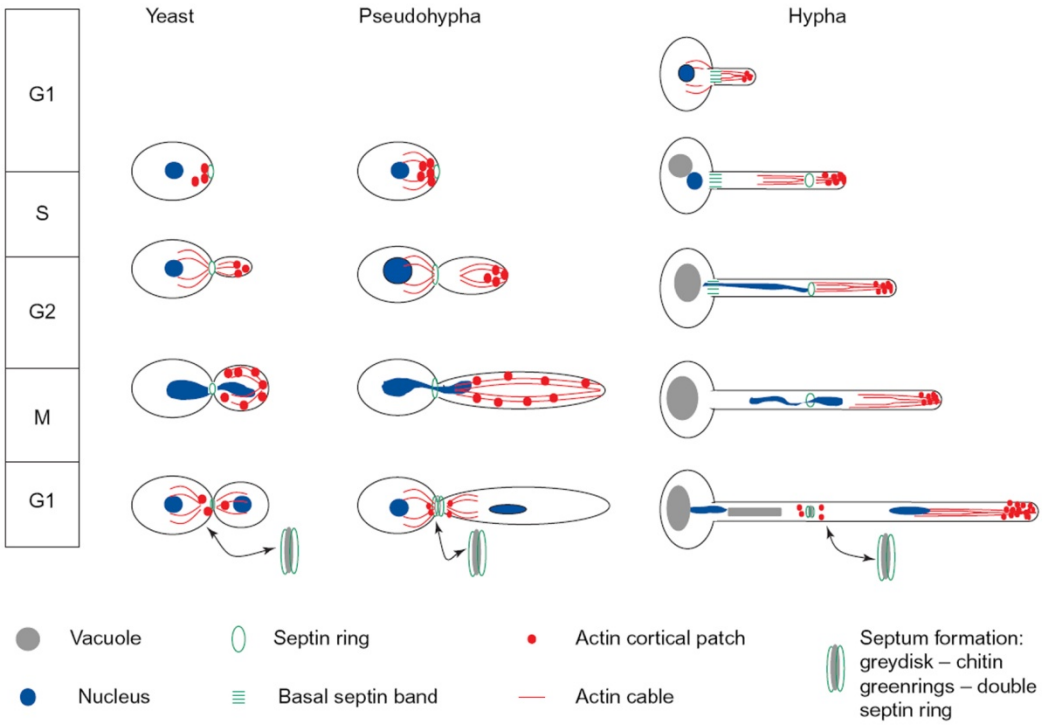

Figure 1. 3 Characteristic features of cell division for the yeast, pseudohyphal, and hyphal growth forms in *C. albicans.*

In the yeast and pseudohyphal growth modes, a bud emerges from the mother cell, defining the position of the first septum. Changes in the pattern of actin polarization reflect a switch from polarized growth at the tip to isotropic growth throughout the growing bud. In pseudohyphal growth, daughter cells become increasingly elongated due to an extended period of polarized growth. Mitosis occurs across the plane of the septum at the mother-daughter junction. In yeast growth, cytokinesis yields two discrete cells while in pseudohyphal growth, cells remain attached, separated by constrictions. In hyphal development, a germ tubes emerges from the mother cell before the G1/S transition. The first septum is formed within the germ tube, where mitosis occurs. One nucleus migrates back into the mother cell, while the other moves further into the germ tube. Actin cortical patches continue to cluster at the growing tip, resulting in an elongated hypha. Hyphal cells remain attached, forming septated filaments. Adapted from Sudbery *et al*. (2004).

1.1.2 Biofilms

C. albicans biofilms are defined as surface-associated microbial communities, encased within a matrix of extracellular polymers (Costerton *et al.*, 1995). Although not a distinct *C. albicans* morphology *per se*, hyphae and pseudohyphae play a determinant role in biofilm development. *C. albicans* biofilms show a complex three-dimensional architecture and display extensive spatial heterogeneity, consisting of a dense network of yeasts, hyphae, and pseudohyphae encased within a matrix of exopolymeric material (Figure 1.4A). Biofilm formation occurs in three distinct stages: (i) attachment and colonization of yeast cells to a surface, (ii) germ tube formation and proliferation of yeast and filamentous cells, which allows the formation of a basal layer of anchoring cells, and (iii) growth of pseudohyphae and hyphae and secretion of a carbohydrate- and protein-rich extracellular matrix (Chandra *et al.*, 2001; Douglas, 2003; Ramage *et al.*, 2001) (Figure 1.4B). While hyphal cells provide the scaffold to build the biofilm, they also express adhesin-encoding genes (discussed in section 1.4.1), which contribute to cell surface adherence properties required to maintain the structural integrity of the biofilm.

C. albicans biofilms have important clinical consequences. They are involved in mucosal candidiasis (thrush, vulvovaginitis), form at the surface of implanted medical devices, such as prostheses, stents, shunts, implants, and various types of catheters, and can cause their failure (Kojic & Darouiche, 2004), have increased antifungal drug resistance, resist host immune defenses, and constitute a sustained reservoir of infecting cells (Lopez-Ribot, 2005).

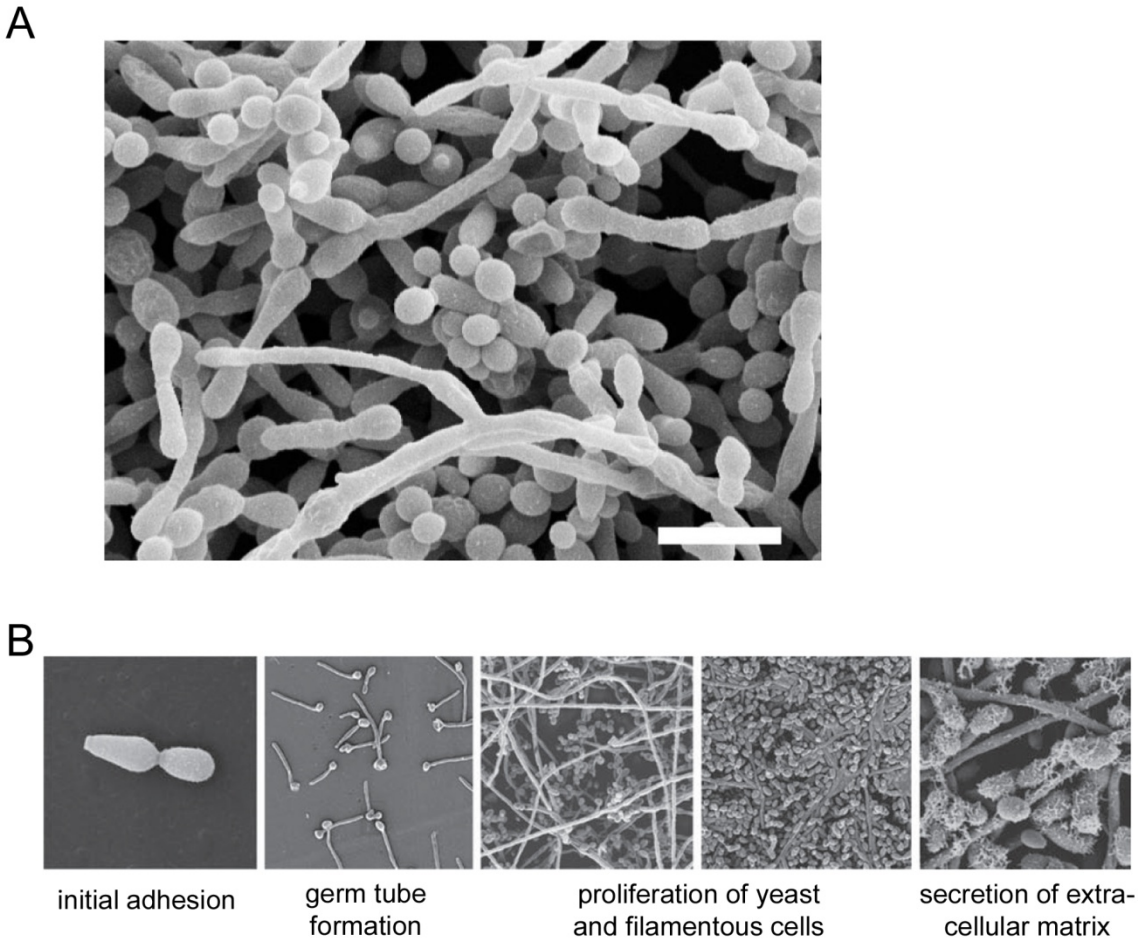

Figure 1. 4 *Candida albicans* biofilms.

(A) Scanning electron microscopy (SEM) image of a mature (48 h) *C. albicans* biofilm, composed of yeast cells, pseudohyphae, and hyphae. Bar = 10 μm. Adapted from Ramage *et al*. (2005). (B) The SEM images show the stages of *C. albicans* biofilm formation, starting with the initial adhesion and attachment of *C. albicans* yeast cells to a surface, followed by the formation of germ tubes. Yeast and filamentous cells proliferate, forming a basal layer of anchoring cells. Further proliferation of microcolonies and filamentation contribute to biofilm development. As the biofilm matures, it becomes encased within a matrix of extracellular material. Adapted from Ramage *et al*. (2009).

1.1.3 Phenotypic switching

A change in cellular morphology also occurs during colony switching, which is seen only in specific strains (Pomes *et al.*, 1985; Slutsky *et al.*, 1985). At low frequency, *C. albicans* strain 3153A can spontaneously and reversibly convert from smooth, white dome-shaped colonies to variant colony shapes in which cells grow in yeast and filamentous forms (star, ring, irregular wrinkle, hat, stipple, and fuzzy) (Berman & Sudbery, 2002). In the *C. albicans* strain WO-1, the white-opaque switch involves the transition from white domed colonies containing white cells to opaque, flat colonies containing opaque cells (Figure 1.2D). White cells are round with a morphology resembling that of Baker's yeast while opaque cells are oblong, twice the size of white cells, and have pimples on the surface of the cell wall (Anderson & Soll, 1987) (Figure 1.2E-F). White and opaque cells differ in virulence and in the capacity to colonize and infect different body locations. Indeed, white cells appear to be better suited for internal infections, while opaque cells thrive in skin infections (Kvaal *et al.*, 1999).

The white-opaque switch has garnered more attention since it was shown to be required for mating (Bennett & Johnson, 2005; Miller & Johnson, 2002). In *C. albicans*, there are two mating-type-like (*MTL*) loci, *MTLa* and *MTLα*, which reside on chromosome 5 and include the *MTL* genes *MTLa1*, *MTLα1*, and *MTLα2* (Hull & Johnson, 1999). For mating to occur, diploid cells have to undergo loss of heterozygosity at the *MTL* loci, resulting in a or α strains which express only *MTLa* or *MTLα* genes. Strains that are homozygous or hemizygous at the *MTL* have a tendency to undergo the white-opaque switch (Lan *et al.*, 2002; Slutsky *et al.*, 1987) (Figure 1.5A). Moreover, opaque cells mate more efficiently (Miller & Johnson, 2002). Diploid a and α opaque cells mate to create tetraploid a/a/α/α cells (Figure 1.5B). Although meiosis has not been observed in *C. albicans*, tetraploid cells undergo chromosome loss under certain laboratory conditions, thereby returning to the diploid state and completing a parasexual cycle (Bennett &

Johnson, 2003). By making the white-opaque switch a prerequisite for mating, *C. albicans* ensures that mating only occurs under specific conditions.

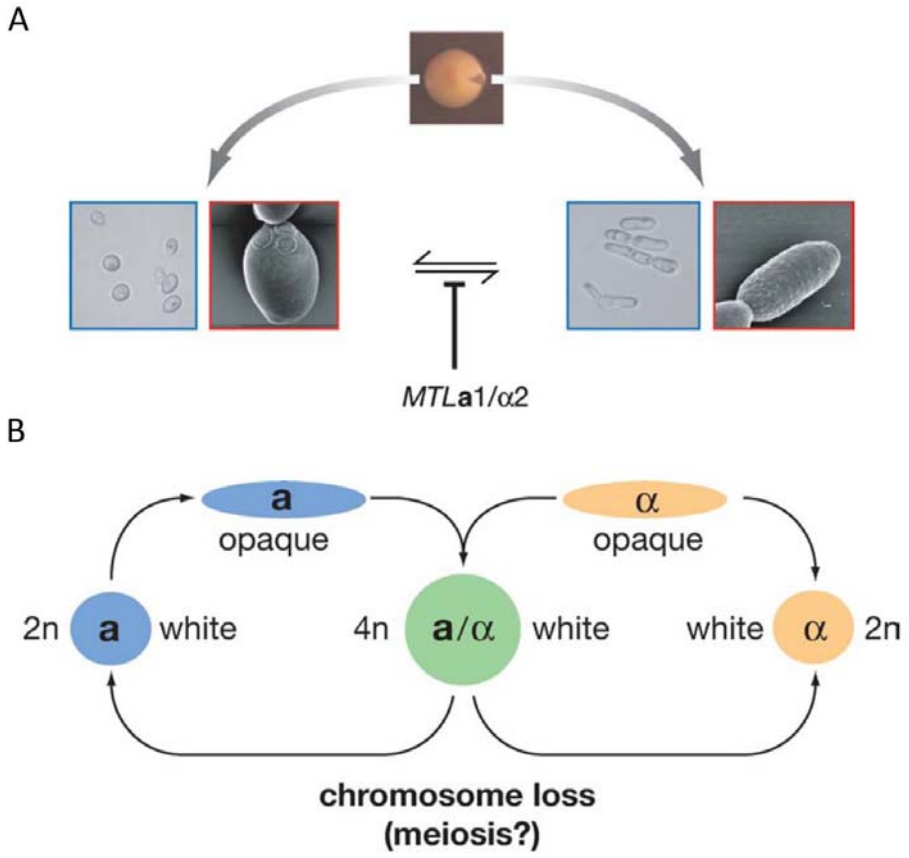

Figure 1. 5 White-opaque switching in *C. albicans*.

(A) A sectored colony on an agar plate, in which most of the cells are in the white phase, but containing a minority of cells in the opaque phase. SEM images show white cells, which are round, and opaque cells, which appear oblong, twice the size of white cells and have pimples on the surface of the cell wall. The transition from the white phase to the opaque phase is blocked by the a1 and α2 proteins, ensuring that a/α strains are not permissive for white-opaque switching. Only a or α strains can switch to the opaque phase, the state in which they are primed to mate. (B) Mating of opaque cells yields a mononuclear tetraploid a/a/α/α cell. Reduction in ploidy can be achieved by chromosome loss, thereby regenerating a- and α–mating competent progeny and completing a parasexual cycle. Meiosis has not been observed in *C. albicans*. Adapted from Bennett & Johnson (2005).

1.1.4 Chlamydospores

Compared to other morphological growth forms, the chlamydospore growth mode remains elusive. Chlamydospores, thick-walled spherical cells that are ~3 to 4 times larger than normal yeast cells, appear to be a dormant growth form (Jansons & Nickerson, 1970) (Figure 1.2G). They have a high lipid and carbohydrate content, and are known to germinate under certain conditions (Fabry *et al.*, 2003; Miller *et al.*, 1974). Chlamydospore formation is induced at low temperature and in conditions of oxygen, light, and nutrient depletion (Staib & Morschhauser, 2007). Media consisting of rice or cornmeal agar supplemented with the detergent Tween 80 have been designed to induce chlamydospore production. This growth mode remains obscure as chlamydospores have rarely been observed during infection and do not appear to play a role in pathogenicity.

1.2 Morphogenetic signals

The yeast-to-hypha transition in *C. albicans* is triggered by a large number of different treatments which are presumed to reflect host conditions encountered by the pathogenic yeast in vivo (Odds, 1988a). Environmental cues that promote hyphal development are presented in Figure 1.6, and include a neutral or alkaline pH (6.5-8.0) and a growth temperature of 37°C (Buffo *et al.*, 1984; Davis, 2003; Shapiro *et al.*, 2009), nitrogen and/or carbon starvation (Csank *et al.*, 1998), embedded growth and/or low oxygen concentration (Brown *et al.*, 1999), and a wide range of chemicals (Odds, 1988a). The most commonly used chemicals that have morphogenetic potential are serum, amino acids (proline, methionine), the amino sugar N-acetylglucosamine, glucose, ammonium, and 5% CO_2/bicarbonate (Biswas & Morschhauser, 2005; Ernst, 2000; Hudson *et al.*, 2004; Klengel *et al.*, 2005; Land *et al.*, 1975b; Maidan *et al.*, 2005b; Mattia *et al.*, 1982; Miwa *et al.*, 2004). Phosphate- or alkane-enriched media induce pseudohyphal growth (Hornby *et al.*, 2004; Sorkhoh *et al.*, 1990).

While serum remains the most potent inducer of hyphal growth, its chemical complexity has hampered the quest to identify its hypha-inducing factors. Initially, it was believed that serum induced hyphal growth by imposing nitrogen starvation, given that it is mainly composed of proteins that constitute an inaccessible source of nutrients until they are hydrolyzed (Brown & Gow, 1999). However, serum still stimulates hyphal growth when combined with a rich source of nutrients such as YPD, indicating that a factor other

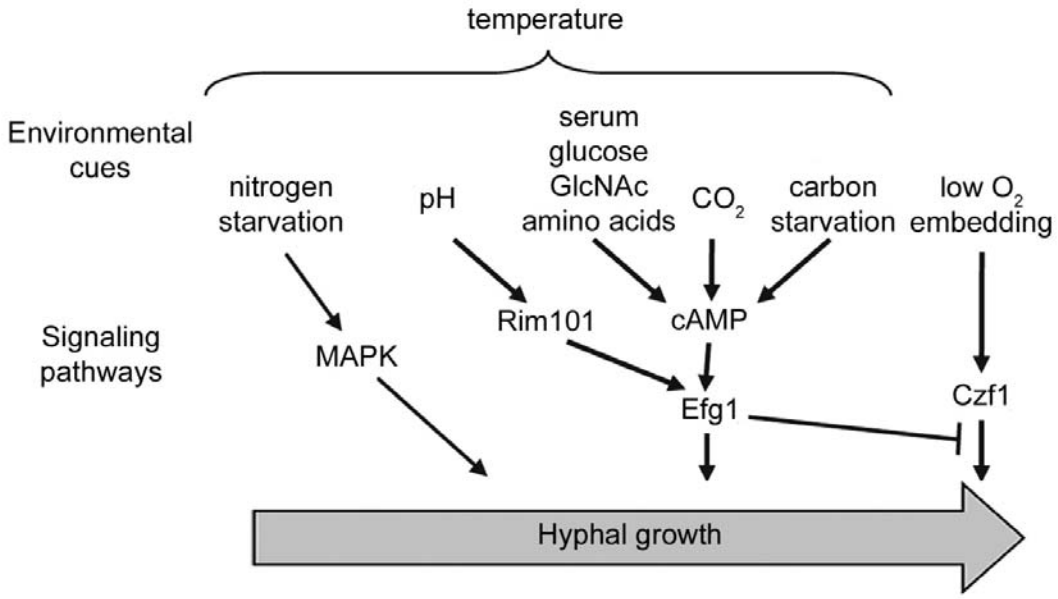

Figure 1. 6 Environmental cues promote hyphal development via specific signaling pathways in *C. albicans*.

Hyphal growth is induced by neutral or alkaline pH, serum, glucose, N-acetylglucosamine (GlcNAc), amino acids, nitrogen and carbon starvation, low oxygen concentration, and embedding in a physical matrix. A high temperature (37°C) is a common requirement for all hypha-inducing conditions, except for embedded growth. Specific signaling pathways mediate the responses to these signals. There appears to be some functional overlap between some of these pathways. Adapted from Brown *et al*. (2007).

than nitrogen starvation is responsible for morphogenesis. Low molecular weight compounds (< 1 kDa) were suggested to be the active factors in serum, as a serum filtrate induced hyphal growth (Feng *et al.*, 1999). Accordingly, Hudson *et al*. (2004) demonstrated that glucose was responsible for ~80% of the serum activity. However, given that hyphal growth is induced using 5-20% serum in media containing glucose at concentrations 10- to 20-fold higher than those found in serum alone, it appears impossible that glucose is the only active agent in serum. Glucose does remain a morphogen, as low concentrations ranging between 0.01% and 0.3% were shown to stimulate hyphal development in liquid and solid media (Hudson *et al.*, 2004; Maidan *et al.*, 2005b). Recently, muramyl dipeptides have been identified as the hypha-inducing factors in serum (Wang & Xu, 2008; Xu *et al.*, 2008).

Although a variety of environmental signals induce hyphal growth, neutral pH and a temperature of 37°C are the two factors that are common to most of the hypha-inducing regimes. A shift from acidic (pH 4.5) to alkaline (pH ≥ 6.5) conditions combined to an increase in temperature is known to promote hyphal growth (Buffo *et al.*, 1984; Lee *et al.*, 1975). Additionally, a distinct pH signaling pathway is responsible for mediating the effects of alkaline pH (Davis, 2009) (see section 1.3.5). In contrast, although it was known that temperature played a pivotal role in triggering morphogenesis, the missing link between high temperature and the onset of hyphal induction was uncovered recently. High temperatures are required to compromise the function of the heat shock protein Hsp90p, a repressor of hyphal growth (Shapiro *et al*., 2009) (discussed in section 3.8).

Different media are routinely used to induce hyphal growth in *C. albicans*. In these media, hypha-inducing cues are a combination of many individual factors, including a high temperature (37°C), a neutral pH, and a low cell density (OD_{600} < 0.1-0.5). Poor carbon sources (e.g. mannitol, N-acetylglucosamine) or low glucose concentrations (e.g. in tissue culture media) impose a state of carbon starvation, which promotes hyphal development (Brown *et al*., 2007). Moreover, hypha-inducing media contain a complex mixture of amino

acids, including proline and methionine, which have morphogenetic properties (Lee *et al.*, 1975; Maidan *et al.*, 2005b; Odds, 1988a). Morphogenesis results from the synergistic effect of a variety of environmental cues (Brown *et al.*, 2007; Ernst, 2000).

1.3 Morphogenetic signaling pathways in *C. albicans*

A complex network of signaling pathways regulate the yeast-to-hypha transition (Figure 1.8). The mitogen-activated protein (MAP) kinase, cAMP-protein kinase A (PKA), Czf1p, Cph2p-Tec1p, and Rim101p pathways stimulate hyphal growth, while the Tup1p signaling pathway represses the yeast-to-hypha transition. Although it was initially thought that each pathway made a somewhat independent contribution in regulating hyphal development, it is now clear that there is cross-talk between pathways. Most of the knowledge pertaining to morphogenetic signaling pathways in *C. albicans* has been gained by examining phenotypes of mutant strains lacking one (or more) specific gene(s). Thus, before describing the role of signaling pathways in morphogenesis, several aspects of *C. albicans* genetics are discussed.

The study of the molecular mechanisms regulating the yeast-to-hypha transition in *C. albicans* has been hindered by genetic features of the pathogenic yeast, including its diploidy, its lack of an exploitable sexual cycle, its alternative codon usage, and the unavailability of good molecular tools and transformation protocols. One approach used to gain insight into biological aspects and gene functions is to analyze strains in which a specific gene is deleted or mutated. Given that *C. albicans* is an obligate diploid organism, a mutation has to become homozygous to observe a pronounced phenotype. In diploid organisms, homozygotes are generated by using genetic cross. However, although *C. albicans* carries a mating-type-like locus (Hull & Johnson, 1999) and can be engineered to mate (Magee & Magee, 2000; Miller & Johnson, 2002) (discussed in section 1.1.3), its

sexual cycle is not exploitable, rendering genetic cross superfluous. Moreover, molecular tools to sequentially disrupt both alleles of a gene in *C. albicans* were initially unavailable.

For these reasons, *C. albicans* genes (involved in morphogenesis or in other biological processes) used to be identified/analyzed based on their ability to complement or suppress mutations in the corresponding *S. cerevisiae* genes (Liu *et al.*, 1994) or to interfere with or constitutively activate the corresponding signaling pathways in *S. cerevisiae* (Whiteway *et al.*, 1992). The sequencing and annotation of the *C. albicans* genome revolutionized research in *C. albicans*: gene functions could be inferred from those described for *S. cerevisiae* genes and functional analyses could be conducted using reverse genetics (Braun *et al.*, 2005). However, comparative genome analyses showed that several *C. albicans* genes, such as those encoding lipases, proteases, and cell wall proteins, had no homologues in *S. cerevisiae*. In addition, functions of homologous genes had diverged. Moreover, it was becoming clear that while specific components of signaling pathways were conserved between both yeasts, molecular mechanisms and environmental cues had often diverged, perhaps due to the fact *C. albicans* had co-evolved with its human host (Biswas *et al*., 2007).

Advances in gene disruption technology have enabled to carry out studies directly in the pathogenic yeast. The Ura blaster method, the first to be developed, relies on homologous recombination to replace the chromosomal copy of a target gene by the disruption cassette whose 5' and 3' ends are homologous to DNA sequences that flank the target gene (Fonzi & Irwin, 1993) (Figure 1.7A). Direct repeats of the hisG sequence from *Salmonella thyphimurium* flank the *C. albicans URA3* gene, enabling intramolecular recombination events. The disruption cassette is transformed into a strain of *C. albicans* auxotrophic for uracil; Ura^+ heterozygous disruption mutants, in which one allele of the target gene is replaced by the *URA3* cassette, are selected on medium lacking uridine. The *URA3* marker is "recycled" by passaging Ura^+ transformants on medium containing 5-fluoroorotic acid (5-FOA) (Alani *et al.*, 1987; Boeke *et al.*, 1984). 5-FOA selects for strains

that delete the *URA3* gene via intramolecular recombination between the hisG flanks. The resulting Ura$^-$ heterozygous strain is transformed again using the *URA3* cassette to disrupt the second allele of the target gene.

Initially, the Ura blaster method was embraced by the *C. albicans* community. Many mutants were created in order to determine the role of genes in virulence. However, mutant strains displayed several problems. First, *URA3* expression is subject to chromosomal positioning (Brand *et al.*, 2004; Cheng *et al.*, 2003b; Lay *et al.*, 1998). Second, high *URA3* expression levels are required for many processes, including morphogenesis, adhesion, and virulence (Brand *et al.*, 2004; Cheng *et al.*, 2003b; Sundstrom *et al.*, 2002b). Thus, virulence defects may be linked either to the deletion of a target gene or to poor *URA3* expression. Moreover, 5-FOA is a significant mutagen that can cause major chromosomal abnormalities (Wellington *et al.*, 2006).

To circumvent these problems, several solutions have been proposed, including reintegrating a copy of *URA3* at a standard locus from which it is consistently expressed (Murad *et al.*, 2000; Sundstrom *et al.*, 2002b), using dominant selectable markers conferring resistance to mycophenolic acid (Staib *et al.*, 1999; Wirsching *et al.*, 2000) and nourseothricin (Reuss *et al.*, 2004; Roemer *et al.*, 2003; Shen *et al.*, 2005), and using auxotrophic markers that do not affect virulence, such as *HIS1*, *LEU2*, and *ARG4* (Noble & Johnson, 2005). New and improved gene disruption tools have also been made available and have been reviewed elsewhere (Noble & Johnson, 2005) (Figure 1.7B).

Furthermore, regulatable promoters, including the glucose repressible *PCK1* and *MAL2* (Backen *et al.*, 2000; Brown *et al.*, 1996; Leuker *et al.*, 1997), the methionine/cysteine-repressible *MET3* promoter (Care *et al.*, 1999), and the tetracycline-repressible promoter system (Nakayama *et al.*, 2000) have been developed to generate conditional null mutants and to conduct functional studies of essential genes. A collection of gene replacement and conditional expression (GRACE) *C. albicans* mutants has also

been generated, in which one allele of a target gene is replaced by the *HIS3* auxotrophic marker while the other allele is placed under the control of the tetracycline-repressible promoter (Roemer *et al*., 2003).

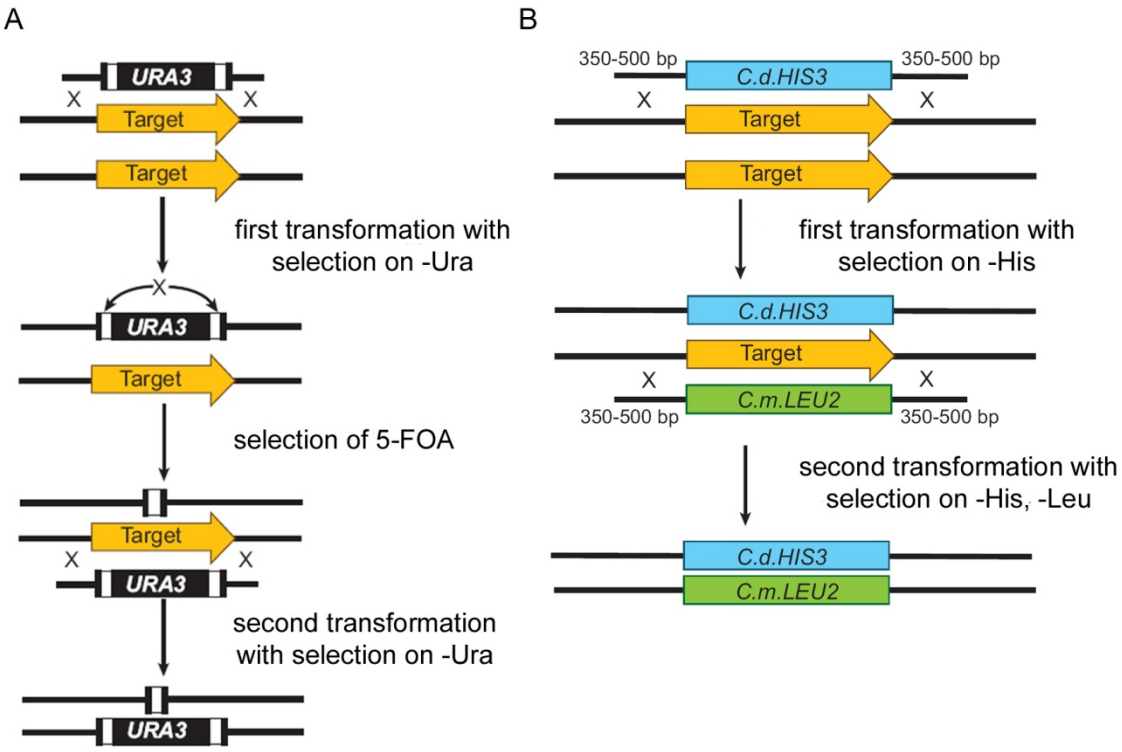

Figure 1. 7 Selected methods for gene disruption in *C. albicans*.

(A) The Ura blaster method consists in transforming a gene disruption cassette into a strain of *C. albicans* auxotrophic for uracil (*ura3*/*ura3*). The cassette harbors the *URA3* gene, flanked by direct repeats of the hisG sequence from *Salmonella typhimurium* and has 5' and 3' ends homologous to sequences flanking the target gene. Mutants in which one allele of the target gene is replaced by the *URA3* cassette are selected on medium lacking uridine. Counterselection on 5-fluoroorotic acid (5-FOA) identifies mutants that have lost the *URA3* sequences through recombination between the hisG repeats. Transformation with the disruption cassette is repeated to disrupt the second copy of the target gene. (B) A linear gene disruption fragment with long regions of homology (300-500 bp) to sequences flanking the target gene is created using a fusion PCR technique. The first allele of a target gene is disrupted by transforming the first disruption fragment marked by the *HIS1* gene from *C. dubliniensis*, and selecting on medium lacking histidine. The second copy of the target gene is disrupted by transforming the second disruption fragment, marked by the *C. maltosa LEU2* gene, and selecting on medium lacking histidine and leucine.

A second issue that has hindered gene function analysis in *C. albicans* is that it displays a noncanonical codon usage: CTG is decoded as a serine instead of a leucine. Consequently, many heterologous markers such as *Escherichia coli lacZ* and jellyfish green fluorescent protein (GFP) cannot be functionally expressed in *C. albicans* unless they are modified (Berman & Sudbery, 2002). To overcome these impediments, a number of specialized reporter genes have been developed (reviewed in Berman & Sudbery [2002]). Convenient cassettes harboring these reporters have been generated, enabling gene expression to be monitored at the RNA or protein level and gene products to be localized in vivo (Gerami-Nejad *et al.*, 2001; Gerami-Nejad *et al.*, 2004; Gerami-Nejad *et al.*, 2009). Thus, the development of molecular tools, combined to the availability of a sequenced genome and to improved transformation protocols, have made it fairly straightforward to conduct functional analyses of signaling genes in *C. albicans*.

1.3.1 The mitogen-activated protein kinase signaling pathway

The first morphogenetic signaling components to be identified in *C. albicans* were members of a MAP kinase pathway, analogous to the filamentous and invasive growth pathways in *S. cerevisiae* (Alonso-Monge *et al.*, 2006; Gimeno *et al.*, 1992; Palecek *et al.*, 2002). In *C. albicans*, the MAP kinase pathway is comprised of three kinases, including Cst20p, homologous to the PAK Ste20p (Csank *et al.*, 1997; Ushinsky *et al.*, 2002), Hst7p homologous to the MAP kinase kinase Ste7p (Leberer *et al.*, 1996), and Cek1p, the *Candida* ERK-like kinase homologous to the MAP kinases Fus3p and Kss1p (Whiteway *et al*., 1992) (Figure 1.8). Although a homologue of the *S. cerevisiae* MAP kinase kinase kinase *STE11* has been annotated (Braun *et al*., 2005), the *C. albicans STE11* gene has not been functionally dissected (Brown *et al*., 2007). These kinases function by sequential phosphorylation, and ultimately activate the transcription factor Cph1p, a homologue of Ste12p which lies downstream of Cek1p (Liu *et al*., 1994). The tyrosine phosphatase Cpp1p is a negative regulator of the MAP kinase, as constitutive hyphal development of a *cpp1/cpp1* mutant was inhibited by deleting *CEK1* (Csank *et al.*, 1997).

The MAP kinase signaling cascade plays a minor role in hyphal growth. Strains lacking *CST20*, *HST7*, *CEK1*, and *CPH1* were unable to form hyphae on solid Spider medium, containing the poor carbon source mannitol and on SLAD nitrogen starvation medium, but filamented in liquid Spider, Lee, and serum media (Csank *et al.*, 1998; Kohler & Fink, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1994). Thus, the MAP kinase induces morphogenesis in response to nutrient limitation on solid media only, while other pathways promote hyphal development in response to other inducing factors.

Activation of the MAP kinase pathway occurs through the GTPase module Cdc42p/Cdc24p (Bassilana *et al.*, 2003) (Figure 1.8). Cdc42p, a Rho-type G protein (Bassilana *et al.*, 2003; Hazan & Liu, 2002) and Cdc24p, its exchange factor, have critical roles in viability and in hyphal growth (Bassilana *et al.*, 2003; Bassilana *et al.*, 2005; Ushinsky *et al.*, 2002; VandenBerg *et al.*, 2004). In response to serum, *CDC24* expression was transiently induced and Cdc24p was recruited to hyphal tips, where it maintained Cdc42p in its GTP-bound active state (Bassilana *et al*., 2005). Cdc42p was shown to bind with high affinity to a Cst20p motif in vitro and to Cla4p, another PAK kinase (Leberer *et al.*, 1997; Su *et al.*, 2005). Given that Ras1p regulates the MAP kinase pathway (Leberer *et al.*, 2001), Bassilana *et al*. (2003) proposed that the Cdc42p/Cdc24p GTPase module formed a complex with Ras1p to activate Cst20p and the MAP kinase pathway. Additionally, in response to nitrogen, the transmembrane ammonium permease Mep2p was shown to be involved in activating the MAP kinase and cAMP-PKA pathways in a Ras1p-dependent manner and in inducing hyphal growth (Biswas & Morschhauser, 2005).

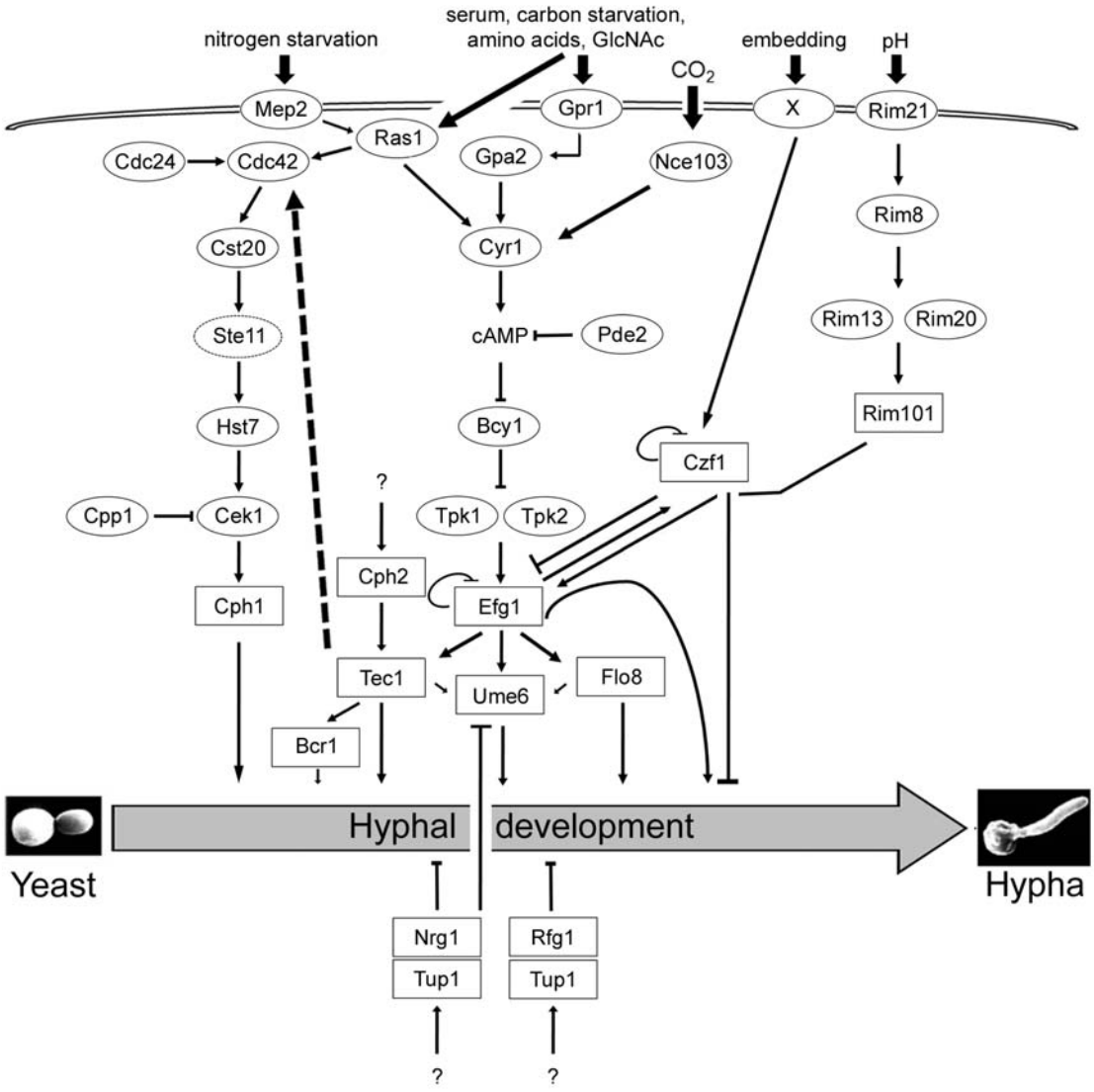

Figure 1. 8 Regulation of morphogenesis in *C. albicans* by multiple signaling pathways.

The MAP kinase, cAMP-PKA, and pH signaling pathways activate morphogenesis, while the yeast-to-hypha transition is repressed by Tup1p, Nrg1p, and Rfg1p. A variety of environmental signals trigger simultaneously different signaling pathways, resulting in the activation of specific transcription factors (boxed) which induce or repress hyphal development. An important regulator of hyphal growth, the small GTPase Ras1p, functions upstream of the MAP kinase and the cAMP-PKA signaling pathways, which operate through the transcription factors Cph1p and Efg1p, respectively. Efg1p appears to act as a hub, as it induces hyphal growth in response to different environmental cues, including serum, carbon starvation, CO_2, and neutral pH. However, Efg1p is a repressor of filamentation in embedded conditions. Hyphal development is repressed by Tup1p, which functions by recruiting the DNA-binding proteins Nrg1p and Rfg1p. Adapted from Brown *et al*. (2007).

Recent data described the mucin-like protein Msb2p and the adaptor protein Sho1p, two plasma membrane proteins, as potential sensors of the MAP kinase pathway (Roman *et al.*, 2009b). During resumption of growth, Cek1p phosphorylation depended on *SHO1* (Roman *et al.*, 2005; Roman *et al.*, 2009a). In addition, Msb2p and Sho1p played a role in hyphal and invasive growth and in the activation of Cek1p in response to cell wall stress (Roman *et al.*, 2005; Roman *et al.*, 2009b). In *S. cerevisiae*, Cdc42p was shown to interact with Msb2p (Cullen *et al*., 2004) and Ste20p (Cst20p homologue) (Peter *et al.*, 1996), while in *C. albicans*, the interaction between Cdc42p and Cst20p was demonstrated (Su *et al*., 2005). Based on these findings, Roman *et al*. (2009b) proposed that a putative Msb2p/Sho1p complex may interact with Cdc42p, promoting the recruitment of Cdc24p and the sequential phosphorylation of Cst20p and Cek1p. However, an interaction between Msb2p and Sho1p has yet to be demonstrated.

The function of the MAP kinase pathway depends on the transcription factor Cph1p, the *S. cerevisiae* Ste12p homologue (Liu *et al*., 1994). Contrary to Ste12p, Cph1p does not regulate the expression of *TEC1*, a transcription factor involved in morphogenesis (discussed in section 1.3.4). *CPH1* was required for the expression of several hypha-specific genes, including *ECE1*, *HWP1*, *HYR1*, *RBT1*, *SAPs5-6*, and *RBT4*, but only in conditions in which hyphal growth depended on the MAP kinase pathway (Lane *et al.*, 2001a). It remains that a *cph1/cph1* mutant strain filamented and expressed hypha-specific genes in most liquid hypha-inducing conditions, thereby questioning the presumed involvement of the MAP kinase pathway in morphogenesis (Braun & Johnson, 2000; Brown *et al.*, 2007; Lane *et al.*, 2001a). In fact, findings suggest that the MAP kinase pathway plays a clear role in mating, rather than in hyphal development (Bennett *et al.*, 2003; Chen *et al.*, 2002; Magee *et al.*, 2002; Sahni *et al.*, 2010).

Although the MAP kinase pathway plays a minor role in morphogenesis and hypha-specific genes expression, it does contribute to a certain extent to *C. albicans* pathogenesis in vivo. Strains lacking *CDC42*, *CDC24*, *CST20*, *CEK1*, and *CPP1* were attenuated in a

mouse model of systemic candidiasis (Bassilana *et al.*, 2003; Csank *et al.*, 1997; Csank *et al.*, 1998; Leberer *et al.*, 1996). Inactivation of *CEK1* and *CPP1* also attenuated virulence in the mammary glands of lactating mice, preventing tissue colonization (Guhad *et al.*, 1998a; Guhad *et al.*, 1998b). However, deletion of *CDC42*, *CDC24*, and *CEK1* impaired growth in vitro, which may account for reduced virulence of mutant strains in vivo. In contrast, *hst7/hst7* and *cph1/cph1* mutant strains retained their virulence in the mouse model of systemic candidiasis (Leberer *et al.*, 1996; Lo *et al.*, 1997). It remains that *CPH1* contributed to the residual virulence of an *efg1/efg1* mutant, suggesting that the MAP kinase is involved in virulence in vivo (Lo *et al*., 1997).

It is noteworthy to mention that like in *S. cerevisiae*, *C. albicans* has several other MAP kinase signaling pathways, including a cell integrity pathway, the protein kinase C (PKC) pathway and a stress-activated kinase pathway (i.e. the HOG pathway), which have been reviewed elsewhere (Alonso-Monge *et al*., 2006).

1.3.2 The cAMP-PKA signaling pathway

The cAMP-PKA signaling pathway plays an important role in filamentation in *S. cerevisiae*, in *C. albicans*, and in other fungi (Lengeler *et al.*, 2000). In the pathogenic yeast, an increase in cAMP accompanies the yeast-to-hypha transition, thereby linking cAMP signaling to filamentation. Components of the cAMP signaling pathway include the G protein-coupled receptor Gpr1p, the Gα protein Gpa2p, the GTPase Ras1p, the adenylate cyclase Cyr1p, the cAMP-dependent PKA, and the transcriptional factor Efg1p (Figure 1.8).

cAMP signaling is regulated by Ras1p, Gpr1p-Gpa2p, and the transmembrane ammonium permease Mep2p. Each upstream regulator responds to a specific set of inducing factors and activates the cAMP signaling pathway. For instance, on solid media or in presence of the amino acid methionine, Gpr1p and Gpa2p were required for

morphogenesis. Moreover, filamentation defects of *gpr1/gpr1* and *gpa2/gpa2* strains were corrected by the addition of exogenous cAMP or by the overexpression of downstream components of the cAMP-PKA pathway, indicating Gpr1p and Gpa2p function upstream of cAMP signaling. However, both mutants were only mildly affected in liquid hypha-inducing media, which suggests that Gpr1p and Gpa2p play minor roles in morphogenesis (Maidan *et al.*, 2005a; Miwa *et al.*, 2004; Sanchez-Martinez & Perez-Martin, 2002). Additionally, in nitrogen starvation conditions, Mep2p induced hyphal growth by activating cAMP signaling in a Ras1p-dependent manner, indicating that the transmembrane permease lies upstream of Ras1p (Biswas & Morschhauser, 2005).

Ras1p belongs to the Ras superfamily of small guanosine triphosphatases (GTPases) (Wennerberg *et al.*, 2005). Ras proteins regulate cell growth, proliferation, and differentiation (Hancock, 2003). In *C. albicans*, Ras1p is required for morphogenesis in most hypha-inducing conditions, suggesting it is a major regulator of the developmental process (Feng *et al.*, 1999; Leberer *et al.*, 2001; Zhu *et al.*, 2009). Ras1p regulates morphogenesis by activating the cAMP-PKA and MAP kinase signaling pathways, given that the addition of exogenous cAMP or the overexpression of components of the MAP kinase cascade corrected the filamentation defect of a *ras1/ras1* mutant (Leberer *et al*., 2001).

As a small GTPase, Ras1p exhibits high-affinity binding for GDP and GTP, and possesses low intrinsic GTP hydrolysis and GDP/GTP exchange activities. Ras1p is activated upon being loaded with GTP by its guanine nucleotide exchange factor (GEF) encoded in *C. albicans* by *CSC25* (*CDC25*). The GTP-bound form of Ras1p possesses high affinity for effector targets, enabling their activation. The GTPase-activating protein (GAP) encoded by *IRA2* accelerates Ras1p's intrinsic GTPase activity, thus promoting the formation of the inactive GDP-bound form. The activation of Ras1p is essential for hyphal growth, as a *cdc25/cdc25* mutant exhibited a filamentation defect (Shapiro *et al.*, 2009; Uhl *et al.*, 2003). In contrast, a strain deleted for *IRA2* was hyperfilamentous (Shapiro *et al*.,

2009). Likewise, a dominant-active Ras1p mutation ($Ras1p^{G13V}$) promoted hyphal growth in noninducing conditions (Feng *et al*., 1999).

Activation of the cAMP signaling pathway occurs upon the synthesis of cAMP, a secondary messenger, by adenylate cyclase. *CYR1*, which encodes the adenylate cyclase in *C. albicans*, contributes to hyphal growth in most solid and liquid hypha-inducing conditions, except in conditions of embedded growth (Cao *et al.*, 2006; Rocha *et al.*, 2001). The morphogenetic growth defect of the *cyr1/cyr1* mutant was corrected by the addition of exogenous cAMP, indicating the importance of Cyr1p in activating cAMP signaling. Moreover, *RAS1* overexpression did not restore filamentation to a *cyr1/cyr1* mutant strain, which suggests that Ras1p lies upstream of Cyr1p (Rocha *et al.*, 2001). Interestingly, activated Ras1p was shown to bind directly to the N-terminal Ras-association (RA) domain of Cyr1p and to promote cAMP synthesis (Fang & Wang, 2006).

Cyr1p can also be activated directly by morphogenetic molecules, including CO_2 and the hypha-inducing factors of serum muramyl dipeptides. CO_2/bicarbonate and muramyl dipeptides induced hyphal growth by stimulating directly the adenylate cyclase catalytic domain and by binding to the leucine-rich repeat domain of Cyr1p, respectively, resulting in cAMP synthesis (Klengel *et al.*, 2005; Xu *et al.*, 2008). These findings demonstrate that factors other than Ras1p contribute to the activation of Cyr1p upon hyphal development. Accordingly, transcript profiling of *ras1/ras1* and *cyr1/cyr1* mutants indicated that Cyr1p regulated a subset of genes independently of Ras1p (Harcus *et al.*, 2004). Thus, Cyr1p is activated by Ras1p, but also by serum components and CO_2 (Klengel *et al.*, 2005; Leberer *et al.*, 2001; Rocha *et al.*, 2001; Xu *et al.*, 2008). In addition, it may also be activated by Gpr1p-Gpa2p (Maidan *et al*., 2005a) (Figure 1.8).

cAMP signaling is positively regulated by the adenylate cyclase-associated protein encoded by *SRV2*. The high-affinity phosphodiesterase encoded by *PDE2* hydrolyzes cAMP to AMP and downregulates cAMP signaling. *SRV2* contributed to hyphal growth in

various solid and liquid inducing conditions and was required for cAMP synthesis (Bahn & Sundstrom, 2001; Bahn *et al.*, 2007). In contrast, a *pde2*/*pde2* mutant was constitutively filamentous, presumably due to increased intracellular cAMP levels (Bahn *et al*., 2003). Upon the yeast-to-hypha transition, the induction of *PDE2* coincides with the gradual decline in cAMP levels (Jung & Stateva, 2003). Recently, Ras2p, an atypical Ras protein, was also shown to negatively regulate cAMP signaling (Zhu *et al*., 2009).

Increasing cAMP levels activate the cAMP-dependent PKA. In *C. albicans*, PKA is constituted of a regulatory subunit and two catalytic subunits. *BCY1* encodes the enzyme's regulatory domain, while *TPK1* (Bockmuhl *et al.*, 2001) and *TPK2* (Sonneborn *et al.*, 2000) encode the catalytic subunits of PKA (Cassola *et al.*, 2004; Staab *et al.*, 2003). cAMP binds to PKA's regulatory domain Bcy1p, causing a conformational change which results in the release and the activation of the catalytic subunits Tpk1p and Tpk2p. Thus, Bcy1p is a negative regulator of PKA, keeping it in an inactive state by inhibiting its phosphotransferase activity. Accordingly, overexpression of *BCY1* blocked hyphal development in solid and liquid media, demonstrating that Bcy1p is a negative regulator of morphogenesis (Staab *et al*., 2003). In contrast, Tpk1p and Tpk2p are positive regulators of the yeast-to-hypha transition, albeit they function in different inducing conditions. For instance, while Tpk1p was required for hyphal growth on solid media only, Tpk2p was necessary for hyphal growth in liquid media and for invasive growth (Bockmuhl *et al.*, 2001; Sonneborn *et al.*, 2000). Additionally, epistasis analysis placed both Tpk1p and Tpk2p downstream of Ras1p, but upstream of the transcription factor Efg1p (Bockmuhl *et al*., 2001).

The cAMP signaling pathway mainly operates through the basic helix-loop-helix (bHLH) transcription factor Efg1p, a member of the APSES family of fungus-specific transcriptional regulators. Hyphal development induced by various signals such as serum, N-acetylglucosamine, proline, Spider medium, neutral pH, and CO_2, depends on Efg1p (El Barkani *et al.*, 2000; Lo *et al.*, 1997; Stoldt *et al.*, 1997). Thus, Efg1p appears to be a hub

that receives input from many environmental cues and signaling pathways (Figure 1.8). Additionally, Efg1p is also involved in chlamydospore formation and phenotypic switching (Sonneborn *et al.*, 1999a; Sonneborn *et al.*, 1999b; Srikantha *et al.*, 2000), while it is a repressor of filamentation induced in microaerophilic or embedded conditions (Giusani *et al.*, 2002) (see section 1.3.3). *EFG1* overexpression corrected filamentation defects of *tpk1/tpk1* and *tpk2*/*tpk2* mutants, placing it downstream of PKA. Yet, this suppressive activity depended on a potential phosphorylation site for PKA (threonine 206) in Efg1p, suggesting that PKA may phosphorylate and activate Efg1p (Bockmuhl *et al*., 2001). So far, the phosphorylation of Efg1p by PKA has not been demonstrated.

Efg1p regulates the expression of genes involved in hyphal growth, cell wall dynamics, virulence, and metabolism (Doedt *et al.*, 2004; Lane *et al.*, 2001a; Sohn *et al.*, 2003). Transcriptional profiling showed that Efg1p regulated the expression of over 200 genes during the yeast-to-hypha transition. Interestingly, approximately 75% of those transcripts were repressed, suggesting Efg1p functions primarily as a transcriptional repressor. Efg1p was shown to bind directly to the promoters of several Efg1p-repressed genes and to its own promoter (Tebarth *et al.*, 2003; Wang *et al.*, 2009). Indeed, *EFG1* is an autoregulated gene, as a decline in *EFG1* transcript levels correlated with the binding of Efg1p to its own promoter (Stoldt *et al.*, 1997; Tebarth *et al.*, 2003). The Sin3p-Rpd3p-containing histone deacetylase complex is involved in the negative autoregulation of *EFG1* (Tebarth *et al*., 2003).

Additionally, Efg1p is also an activator of several hypha-specific genes, including *ALS3*, *HWP1*, and *ECE1* (Braun & Johnson, 2000; Leng *et al.*, 2001; Sharkey *et al.*, 1999). The binding of Efg1p to the promoters of these genes was shown to be required for the recruitment of the Swi/Snf chromatin remodeling complex, enabling their transcriptional activation during hyphal growth (Lu *et al.*, 2008). Transcriptional profiling also revealed that Efg1p regulated functions that were unrelated to hyphal development, including the expression of yeast cell wall genes and of metabolic genes (Doedt *et al.*, 2004; Harcus *et*

al., 2004; Sohn *et al.*, 2003). Like other APSES proteins, Efg1p functions as an inducer and a repressor of gene expression, whose activity is determined by interacting with auxiliary proteins. One of these proteins is Flo8p, a transcriptional activator required for hyphal growth induced in various conditions (Cao *et al*., 2006). Transcriptional profiling showed that Flo8p controlled a subset of hypha-specific, Efg1p-regulated genes. Moreover, Flo8p was shown to interact with Efg1p using a two-hybrid assay and by immunoprecipitation, suggesting that Flo8p functions with Efg1p to regulate morphogenesis (Cao *et al*., 2006) (Figure 1.8).

The cAMP signaling pathway plays a significant role in virulence, as it controls the expression of many genes encoding virulence factors (Sundstrom *et al.*, 2002a; Zhao *et al.*, 2004). Indeed, strains lacking components of the cAMP pathway, including Ras1p, Cyr1p, Srv2p, Pde2p, Efg1p, and Flo8p, were compromised in virulence in a mouse model of systemic candidiasis (Bahn & Sundstrom, 2001; Bahn *et al.*, 2003; Cao *et al.*, 2006; Leberer *et al.*, 2001; Lo *et al.*, 1997; Rocha *et al.*, 2001). In addition, both *tpk2/tpk2* and *efg1/efg1* mutants were attenuated in virulence in a mouse model of oropharyngeal candidiasis (Park *et al.*, 2005), while only the *efg1/efg1* mutant was unable to cause lethality in an immunodeficient mouse model of oro-esophageal candidiasis (Westwater *et al.*, 2007). These findings demonstrate the role of the cAMP pathway in pathogenesis. However, cAMP signaling is not involved in all types of *Candida* infections, as an *efg1/efg1* mutant retained its virulence in an infection model that reflects hypha induction via physical contact (i.e. embedded growth) (Riggle *et al.*, 1999).

1.3.3 The Czf1p signaling pathway

Filamentation in embedded conditions is induced in response to physical cues in the environment rather than to chemical cues such as pH, morphogens, or starvation (Brown *et al*., 1999). The transcription factors Czf1p, Efg1p, and Flo8p regulate filamentous growth in embedded conditions, as a *czf1/czf1* mutant strain was compromised for filamentation

while *efg1/efg1* and *flo8/flo8* mutants were hyperfilamentous (Brown *et al.*, 1999; Cao *et al.*, 2006). Thus, filamentation in these conditions is positively and negatively regulated by Czf1p and the cAMP signaling pathway, respectively (Figure 1.8).

Several lines of evidence indicate that a complex autoregulatory loop involving *CZF1* and *EFG1* regulates filamentation in embedded conditions, whereby Czf1p promotes filamentation by antagonizing Efg1p function (Giusani *et al.*, 2002). First, Efg1p is a repressor of filamentation induced in embedded conditions (Giusani *et al.*, 2002). In addition, Efg1p and Czf1p were shown to interact physically in the yeast two-hybrid system (Giusani *et al.*, 2002). Efg1p was also shown to bind the *CZF1* promoter and was required for its expression in embedded conditions, suggesting Efg1p is an activator of *CZF1* (Vinces *et al*., 2006). Moreover, *CZF1* is an autoregulated gene, as its overexpression downregulated its transcript levels (Vinces *et al*., 2006). The current model reflecting these findings suggests that in embedded conditions, *CZF1* expression is induced in an Efg1p-dependent manner (Vinces *et al*., 2006). Increased Czf1p levels antagonize Efg1p function, thereby relieving Efg1p-mediated repression of filamentation. Czf1p negatively regulates its own expression by binding to its promoter close to the Efg1p-binding site, which blocks Efg1p-mediated activation of *CZF1*. A decline in Czf1p levels may enable Efg1p to regain activity. Cyclical modulations of Czf1p and Efg1p levels appear necessary for filamentation in embedded conditions (Vinces *et al*., 2006).

1.3.4 The Cph2p-Tec1p signaling pathway

An additional signaling pathway is defined by the transcription factors Cph2p and Tec1p (Figure 1.8). *CPH2* encodes a Myc family bHLH transcription factor required for hyphal growth on solid Lee's and serum-containing media and in liquid Lee's medium (Lane *et al*., 2001b). The function of Cph2p in hyphal growth is mediated in part through Tec1p, as ectopic expression of *TEC1* corrected the filamentation defect of a *cph2*/*cph2* mutant strain. Additionally, Cph2p was required for *TEC1* expression in Lee's medium,

and it was shown to bind to two sterol regulatory element-1-like sequences and to the E-box motif in the *TEC1* promoter, suggesting Cph2p is an activator of *TEC1* (Lane *et al*., 2001b). Epistasis analysis revealed that Cph2p functions independently of the MAP kinase and cAMP signaling pathways (Lane *et al*., 2001b). The upstream regulators of Cph2p have not been identified yet, but casein kinase II, PKC, and PKA may be involved in its activation via phosphorylation. Cph2p appears to play minor roles in virulence (Chamilos *et al.*, 2009; Noble *et al.*, 2010).

Tec1p is a member of the TEA/ATTS family of transcription factors which recognize and bind to the consensus sequence 5' CATTCY 3' in target promoters via the TEA DNA-binding region. The *S. cerevisiae* Tec1p homologue functions with Ste12p to control filamentation and invasive growth of haploid and diploid strains, respectively (Madhani & Fink, 1997). In *C. albicans*, Tec1p is involved in hyphal growth, expression of hypha-specific genes, virulence, and biofilm formation (Homann *et al.*, 2009; Nobile & Mitchell, 2005; Nobile *et al.*, 2006a; Schweizer *et al.*, 2000). A *tec1/tec1* mutant strain was attenuated in several models of infection (Chamilos *et al.*, 2009; Fuchs *et al.*, 2010; Schweizer *et al.*, 2000) and in a model of systemic infection, despite the fact the mutant strain filamented. These results suggest that Tec1p is involved in virulence-related activities other than hyphal growth.

In response to hypha-inducing signals, *TEC1* transcript and protein levels increased rapidly (Schweizer *et al.*, 2000; Shareck *et al.*, 2011) (Figure 4.12). *TEC1* induction depended in part on Efg1p, while *TEC1* overexpression partially restored filamentation to an *efg1/efg1* mutant, indicating Tec1p functions in part downstream of Efg1p (Lane *et al.*, 2001a). Conversely, *EFG1* overexpression did not correct the filamentation defect in a *tec1*/*tec1* mutant strain and Tec1p did not regulate *EFG1* transcription (Schweizer *et al.*, 2000). Furthermore, in response to serum, *TEC1* was required for the induction of *CDC24*, which suggests there may be a positive feedback loop between Tec1p and the Cdc24p-Cdc42p branch of the MAP kinase pathway (Bassilana *et al*., 2005).

TEC1 was required for the expression of *BCR1*, which encodes a C_2H_2 zinc finger protein required for biofilm formation and for the expression of hypha-specific adhesins such as *HYR1*, *HWP1*, *RBT5*, *ALS3*, and *ALS1* (Nobile & Mitchell, 2005; Nobile *et al.*, 2006a). Thus, Bcr1p functions downstream of Tec1p (Figure 1.8). Interestingly, *BCR1* was not required for hyphal growth, and although it promoted biofilm formation in vivo, it was not required for virulence in two models of infection (Chamilos *et al.*, 2009; Nobile *et al.*, 2006a).

1.3.5 The Rim101p pH signaling pathway

A pH-responsive pathway enables the pathogenic yeast to sense, respond, and adapt to external pH. The Rim101p signaling pathway, studied extensively in other fungi, including *A. nidulans* and *S. cerevisiae*, has been reviewed elsewhere (Davis, 2003; Davis, 2009; Penalva *et al.*, 2008). In *C. albicans*, the Rim101p signaling pathway not only controls pH-regulated gene expression, but also pH-induced morphogenesis, whereby acidic and alkaline conditions favor yeast growth and hyphal growth, respectively (Davis, 2003). Components of the pathway include the Rim101p transcription factor, Rim21p, Dfg16p, Rim20p, Rim13p, and Rim8p (Davis, 2003) (Figure 1.9). Deletion of *RIM101* or of any gene required for the activation of Rim101p inhibited pH-dependent gene expression and hyphal growth induced at alkaline pH (Davis *et al.*, 2000b; Li *et al.*, 2004).

The current model for the Rim101p signaling pathway suggests that environmental pH is sensed by the plasma membrane receptor proteins Dfg16p and Rim21p (Barwell *et al.*, 2005; Calcagno-Pizarelli *et al.*, 2007). At neutral-alkaline pH, these sensor proteins are stimulated, thereby triggering the ubiquitination of the arrestin-like protein Rim8p and promoting endocytosis (Herranz *et al.*, 2005). The endosomal-sorting complex required for trafficking (ESCRT) protein module is recruited (Xu *et al.*, 2004). Rim13p and Rim20p are also recruited and brought in proximity of the inactive full-length transcription factor

Rim101p (Boysen & Mitchell, 2006; Li *et al.*, 2004). The calpain-like protease Rim13p cleaves the inhibitory C-terminal domain of Rim101p, while Rim20p is thought to interact with Rim101p, acting as a scaffold to facilitate the proteolytic cleavage (Kullas *et al.*, 2004). Once activated, Rim101p translocates into the nucleus, where it regulates pH-dependent genes (Li *et al.*, 2004).

Transcriptional profiling revealed that Rim101p regulates the expression of genes involved in hyphal development, cell wall structure, virulence, and iron acquisition (Bensen *et al.*, 2004; Nobile *et al.*, 2008; Thewes *et al.*, 2007). However, expression of the hypha-specific gene *HWP1* was not abolished in a *rim8/rim8* mutant. This is due to the Rim101p pathway signaling through Efg1p (El Barkani *et al.*, 2000) (Figure 1.8). As for pH-dependent genes, Rim101p functions as an activator of alkaline-induced genes and a repressor of alkaline-repressed genes (i.e. *PHR2*) by binding directly to respective promoters (Baek *et al.*, 2006; Ramon & Fonzi, 2003). Moreover, Rim101p binds its own promoter which contains multiple Rim101p consensus sequences (Ramon *et al.*, 1999).

The Rim101p signaling pathway is required for *C. albicans* pathogenesis. Strains deleted for *RIM101* and *RIM8* were attenuated in virulence in a model of systemic candidiasis (Davis *et al.*, 2000a). In addition, *RIM101* and *RIM13* were required for virulence in models of oropharyngeal candidiasis and keratomycosis, respectively (Mitchell *et al.*, 2007; Nobile *et al.*, 2008).

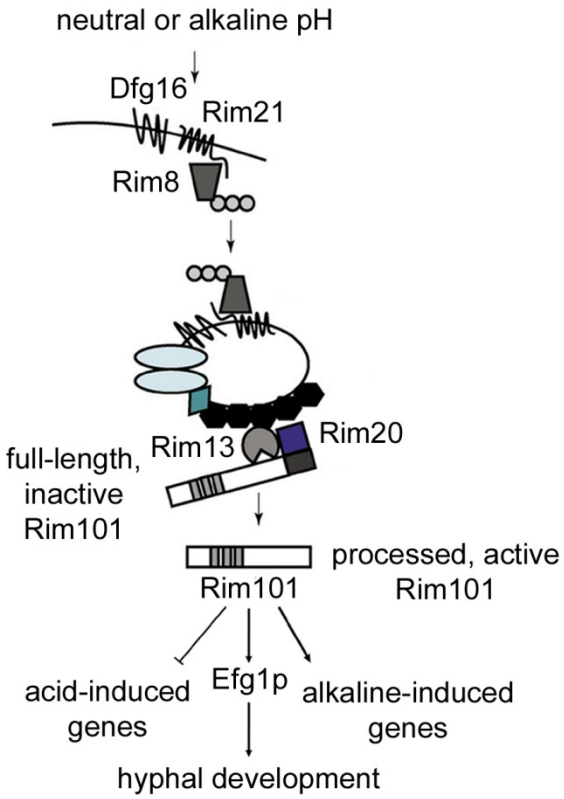

Figure 1. 9 The Rim101p pH signaling pathway in *C. albicans*.

In neutral-alkaline environments, sensors Rim20p and Dfg16p are stimulated, leading to the ubiquitination of Rim8p. The endosomal-sorting complex required for trafficking (ESCRT) protein module is recruited as well as Rim20p and Rim13p. The calpain-like protease Rim13p cleaves off the inhibitory C-terminal region of Rim101p, thereby activating Rim101p. Processed Rim101p activates alkaline-induced genes and represses acid-induced genes. Rim101p operates via Efg1p to promote hyphal development in response to alkaline pH. Adapted from Davis (2009).

1.3.6 The Tup1p signaling pathway

In the absence of morphogenetic signals, the yeast-to-hypha transition is repressed by the Tup1p signaling pathway (Figure 1.8). Tup1p is a repressor of filamentous growth and of hypha-specific gene expression, as its deletion resulted in constitutively filamentous cells in which several hypha-specific genes were derepressed (Braun & Johnson, 1997; Braun *et al.*, 2000; Sharkey *et al.*, 1999). In *S. cerevisiae*, Tup1p represses the transcription of various genes by forming a repressor complex with the corepressor Ssn6p (Smith & Johnson, 2000). This complex is recruited to the promoters of target genes by sequence-specific DNA-binding proteins. In *C. albicans*, the N-terminal domain of Tup1p enables interactions with the corepressor Ssn6p while the seven C-terminal conserved WD40 repeats promote interactions with DNA-binding proteins.

In *C. albicans*, Nrg1p and Rfg1p are two DNA-binding proteins which function with Tup1p to negatively regulate hyphal growth and hyphal gene expression (Braun & Johnson, 1997; Braun *et al.*, 2001; Kadosh & Johnson, 2001; Khalaf & Zitomer, 2001; Murad *et al.*, 2001a; Murad *et al.*, 2001b). In a *nrg1/nrg1* mutant, pseudohyphal growth and hyphal gene expression were derepressed (Braun *et al.*, 2001; Garcia-Sanchez *et al.*, 2005; Kadosh & Johnson, 2005; Murad *et al.*, 2001a; Murad *et al.*, 2001b). Similarly, several hyphal genes were derepressed in a *rfg1/rfg1* mutant, but the hyperfilamentous phenotype was not as dramatic as that seen in a *nrg1/nrg1* mutant (Kadosh & Johnson, 2001; Khalaf & Zitomer, 2001).

NRG1 encodes a sequence-specific DNA-binding protein of the zinc finger family. It recognizes and binds to the Nrg1p response element (NRE) (A/C)(A/C/G)C_3T in target promoters (Murad *et al*., 2001b). Rfg1p, a high mobility group protein, is a homologue of the *S. cerevisiae* hypoxic regulator Rox1p (Kadosh & Johnson, 2001; Khalaf & Zitomer, 2001). In *S. cerevisiae*, the relief of Tup1p repression is achieved by inactivating or removing DNA-binding proteins (Smith & Johnson, 2000). In *C. albicans*, Tup1p repression is relieved by downregulating *NRG1* transcript levels at the onset of the yeast-to-

hypha transition, which enables hyphal development and expression of hypha-specific genes (Banerjee *et al.*, 2008; Braun *et al.*, 2001). Recent findings also showed that Nrg1p activity was regulated at the posttranslational level (Brown *et al*., 2007). It is not clear how Rfg1p is modulated, although there is evidence it may also be regulated at the posttranslational level (Kadosh & Johnson, 2001).

Findings by Kadosh & Johnson (2005) revealed that the relief of Tup1p-mediated repression played a key role in the onset of the hyphal growth program. In addition, while Nrg1p and Rfg1p were shown to function mostly through Tup1p, Tup1p regulated several genes independently of both DNA-binding proteins (Kadosh & Johnson, 2005). Moreover, Ssn6p was not required for the repression of most Tup1p- and Nrg1p-regulated genes (Garcia-Sanchez *et al*., 2005). Although this does not exclude the possibility that Ssn6p is present in Tup1p-containing repression complexes, Tup1p repression appears to be mediated mainly by Nrg1p and, to a lesser extent, by Rfg1p (Brown *et al*., 2007).

Tup1, Nrg1p, and Rfg1p were required for virulence in a mouse model of systemic candidiasis (Braun *et al.*, 2000; Braun & Johnson, 2000; Kadosh & Johnson, 2001; Kadosh & Johnson, 2005; Murad *et al.*, 2001b). Such results appear incongruous, given that hypha-specific genes, many of which encoded virulence factors, were derepressed in strains deleted for each of these repressors. Two reasons may account for these findings. First, compared to parental strains, all three mutant strains had slower growth rates in vitro, which may have affected their growth rates in vivo (Kadosh & Johnson, 2001; Murad *et al.*, 2001a). Second, constitutively filamentous cells may not be disseminated efficiently in systemic models of infection when cells are inoculated directly into the bloodstream (Gow *et al.*, 2002). Indeed, aberrant trafficking accounted for the apparent attenuation in virulence of a *nrg1/nrg1* mutant in a model of systemic candidiasis (Nobile *et al.*, 2008). Moreover, a *nrg1/nrg1* mutant exhibited normal virulence in a model of oropharyngeal candidiasis, in which cells are applied directly to the oral mucosa (Nobile *et al*., 2008). In addition, the depletion of *NRG1* correlated with heightened virulence, while its

overexpression attenuated virulence in a model of systemic candidiasis (Saville *et al.*, 2003; Saville *et al.*, 2006). Thus, these findings question the attenuation in virulence of strains lacking *TUP1*, *NRG1*, and *RFG1*.

1.3.7 The transcription factor Ume6p

In *C. albicans*, *UME6* encodes a zinc finger DNA-binding protein involved in morphogenesis. *UME6* was not required for germ tube formation, but it was essential for hyphal extension under a variety of hypha-inducing conditions (Banerjee *et al.*, 2008; Zeidler *et al.*, 2009). *UME6* expression was induced in a Ras1p-, Cph1p-, Efg1p-, Flo8p- and Tec1p-dependent manner, suggesting that it is a downstream target of these morphogenesis regulators (Zeidler *et al*., 2009). Accordingly, ectopic overexpression of *UME6* rescued the filamentation defect of *ras1/ras1* and *cph1/cph1 efg1/efg1* mutant strains, placing it downstream of the MAP kinase and cAMP-PKA signaling pathways (Figure 1.8). Conversely, *UME6* was derepressed in strains lacking *TUP1*, *NRG1*, and *RFG1*, indicating the transcription factor is also a target of the Tup1p signaling pathway (Banerjee *et al.*, 2008; Kadosh & Johnson, 2005; Zeidler *et al.*, 2009). *UME6* was required for the expression of hypha-specific genes, including *HWP1*, *ECE1*, *RBT1*, *RBT4*, *HYR1*, *PHR1*, and *HGC1* (Banerjee *et al.*, 2008; Carlisle *et al.*, 2009; Zeidler *et al.*, 2009) and played a role in virulence in a mouse model of systemic candidiasis (Banerjee *et al.*, 2008; Carlisle *et al.*, 2009; Zeidler *et al.*, 2009).

The role of *UME6* in filamentation has further been described. The transcriptional regulator was shown to control the level and duration of the hypha-specific G1 cyclin *HGC1*, the only hypha-specific gene required for hyphal growth (Carlisle & Kadosh, 2010; Zheng & Wang, 2004) (see section 1.4.3). In response to hypha-inducing signals, *UME6* is induced as a result of the activation of the cAMP signaling cascade and the relief of transcriptional repression exerted by Tup1p-Nrg1p. Once induced, Ume6p is involved in regulating *HGC1* expression, either by binding directly to the *HGC1* promoter and

directing its transcription or by stabilizing the *HGC1* transcript (Carlisle & Kadosh, 2010). In addition, Ume6p cellular levels were shown to determine morphology in *C. albicans*, whereby high *UME6* expression levels drove hyphal growth, while moderate levels yielded pseudohyphal cells. These findings demonstrate that the pseudohyphal morphology is an intermediate morphological state between yeast and hyphal morphologies (Carlisle *et al*., 2009) (see section 1.1.1).

1.4 Downstream targets of morphogenetic signaling pathways

A virulence factor is defined as "an attribute whose absence in the fungus eliminates or reduces (…) damage in a defined host system in vivo" (Odds *et al*., 2007). In *C. albicans*, virulence factors vary according to the type, site and stage of infection, and the nature of the host response. They include factors that promote adhesion to host cells or to host cell proteins and that degrade and destroy host tissue barriers, thereby enabling invasion. Morphogenesis also contributes to overall virulence. Indeed, *C. albicans* has devised a strategy to co-regulate the yeast-to-hypha transition with the expression of many of its virulence factors (Kumamoto & Vinces, 2005). By coordinating hyphal development and the production of virulence factors, *C. albicans* maximizes its pathogenic properties and activities, thereby ensuring its success as a pathogen.

It is noteworthy to mention another virulence attribute of *C. albicans*, namely its ability to adapt metabolically, to survive and to thrive in a multitude of different niches (Kumamoto, 2008). For instance, upon phagocytosis by neutrophils or macrophages, *C. albicans* switches from a fermentative to an oxidative metabolism (Fradin *et al.*, 2007; Lorenz *et al.*, 2004). Likewise, phenotypic swiching enables cells to survive in harsh environments and evade the innate immune system. Stress response is another means by which *C. albicans* counteracts host immunological defenses. For the purpose of this review,

only hypha-specific virulence factors are discussed, as these factors are downstream targets of morphogenetic signaling pathways (discussed in section 1.3).

The yeast-to-hypha transition also coincides with a change in cell polarity, cell cycle, and cell wall biosynthesis. The machinery that orchestrates these changes is controlled by a set of cyclin-dependent kinases (CDKs) which operate by associating with various cyclins, including the hypha-specific G1 cyclin Hgc1p (Whiteway & Oberholzer, 2004; Zheng & Wang, 2004; Zheng *et al.*, 2007). Because they are downstream outputs of morphogenetic pathways, Hgc1p and the CDK Cdc28p are discussed and their roles in morphogenesis are briefly reviewed.

1.4.1 Virulence factors

1.4.1.1 Adhesins

Adhesins are molecules that promote the adherence of *C. albicans* cells to host cells or host-cell ligands (Calderone & Fonzi, 2001; Sundstrom, 1999). The most notable adhesins in *C. albicans* are encoded by *HWP1* and genes of the *ALS* family. In addition, *RBT1* encodes a GPI-anchored cell wall protein similar to Hwp1p. *HWP1*, *RBT1*, *ALS1*, and *ALS3* are expressed exclusively upon the yeast-to-hypha transition (Bastidas *et al.*, 2009; Hoyer *et al.*, 1998; Staab *et al.*, 1996). While *ALS1*, *ALS3*, and *HWP1* are downstream effectors of transcription factors Efg1p, Bcr1p, and Tec1p (Argimon *et al.*, 2007; Nobile & Mitchell, 2005; Schweizer *et al.*, 2000), *RBT1* is positively regulated by Efg1p and Rim101p (Bensen *et al.*, 2004; Sohn *et al.*, 2003). Repressors Tup1p, Nrg1p, and Rfg1p are also involved in regulating expression of these adhesins (Braun *et al.*, 2001; Garcia-Sanchez *et al.*, 2005; Kadosh & Johnson, 2001; Kadosh & Johnson, 2005; Murad *et al.*, 2001a; Murad *et al.*, 2001b).

HWP1 encodes a hypha-specific cell wall mannoprotein whose N-terminal domain resembles mammalian transglutaminase substrates and is surface-exposed while its C-terminal domain is cross-linked to the cell wall (Staab *et al.*, 1996). A *hwp1/hwp1* mutant strain was less adhesive to oral epithelial cells and was attenuated in virulence in models of oropharyngeal and systemic infections, indicatig that *HWP1* plays a role in adherence to host cells and in virulence (Staab *et al.*, 1999; Sundstrom *et al.*, 2002b). By mimicking host cell proteins, Hwp1p enables *C. albicans* cells to be cross-linked to host oral epithelial cells (Staab *et al.*, 1999). *HWP1* is also involved in cell-cell interactions that are essential for biofim formation (Nobile *et al.*, 2006a; Nobile *et al.*, 2006b). *HWP1* is expressed in vivo, as its transcripts were detected in *C. albicans* clinical samples originating from oral and vaginal infections (Cheng *et al.*, 2003a; Naglik *et al.*, 2006). As for *RBT1*, it was shown to be required for virulence in two models of infection (Braun *et al.*, 2000).

The *ALS* (agglutinin-like sequence) gene family encodes eight cell-surface GPI-anchored proteins required for adherence of *C. albicans* cells to host tissues. The gene product of *ALS1* mediates adherence to human umbilical vein endothelial cells (HUVEC), but not to buccal epithelial cells (BEC) (Zhao *et al.*, 2004) and is essential for virulence in a model of systemic infection (Calderone & Fonzi, 2001). In addition, Als3p is involved in adherence to BEC and to HUVEC (Zhao *et al*., 2004) and in biofilm formation (Nobile & Mitchell, 2005). Both Als1p and Als3p are required for cell-cell adherent interactions during biofilm formation and mediate cellular aggregation in response to rapamycin treatment (Bastidas *et al.*, 2009; Nobile *et al.*, 2006a). Individual *ALS* genes are expressed in vivo, as several *ALS* transcripts were detected in clinical samples and in various models of infection (Cheng *et al.*, 2005; Green *et al.*, 2004; Green *et al.*, 2006).

1.4.1.2 Extracellular hydrolytic enzymes

C. albicans produces extracellular hydrolytic enzymes, including secreted aspartic proteases (SAPs), phospholipases, and lipases, which have been reviewed elsewhere

(Hruskova-Heidingsfeldova, 2008; Naglik *et al.*, 2004; Schaller *et al.*, 2005). At least ten different *SAP* genes encode preproenzymes that are cleaved during transport through the secretory pathway (Naglik *et al.*, 2003). SAPs are involved in the degradation of host barriers during tissue invasion and the destruction of host defence molecules or nutrient supply (Naglik *et al.*, 2003). *C. albicans* mutants in which various *SAP* genes were disrupted had a reduced capacity to damage vaginal and oral epithelial cells in some in vitro models, but not in others (De Bernardis *et al.*, 1999; Hube *et al.*, 1997; Lermann & Morschhauser, 2008; Naglik *et al.*, 2008; Schaller *et al.*, 1999). In these mutants, there appears to be a compensatory mechanism whereby some *SAP* genes are upregulated as a means to compensate for the disruption of other *SAP*s (Correia *et al.*, 2010; Lermann & Morschhauser, 2008). In addition, *SAP* gene expression varies according to growth conditions and to types and stages of *Candida* infections (Hube *et al.*, 1994; White & Agabian, 1995). Only *SAP4*, *SAP5*, and *SAP6* are expressed in hypha-specific Efg1p- and Tec1p-dependent fashions (Felk *et al.*, 2002; Schweizer *et al.*, 2000). In vivo, *SAP5* and *SAP9* are the most highly expressed proteinase genes (Naglik *et al*., 2008).

The term phospholipase (PL) describes a heterogeneous group of enzymes capable of hydrolyzing one or more ester linkages in phospholipids, the major component of biological membranes (discussed in section 1.7.1). In *C. albicans*, PLs are involved in damaging cell membranes and invading host cells, and differ in terms of their modes of action and their targets within a phospholipid molecule. Four subclasses exist, including PLAs (Banno *et al.*, 1985), PLBs (Barrett-Bee *et al.*, 1985; Hoover *et al.*, 1998), PLCs (Pugh & Cawson, 1977), and PLDs (Hube *et al.*, 2001). *C. albicans* harbors two genes coding for PLBs, three for PLCs and one for PLD. *PLB1* and *PLB5* encode secreted phospholipase B proteins which were required for virulence in a model of systemic infection (Leidich *et al.*, 1998; Mukherjee *et al.*, 2001; Theiss *et al.*, 2006). Similarly, *PLD1* was essential for virulence in two mouse models of oral candidiasis, but not in an in vitro model of oral candidiasis (Hube *et al.*, 2001). *PLB1*, *PLB5*, and *PLD1* are expressed

during the yeast-to-hypha transition (Goyard *et al.*, 2008; Hube *et al.*, 2001; Theiss *et al.*, 2006). *PLD1* also contributes to hyphal growth on solid medium.

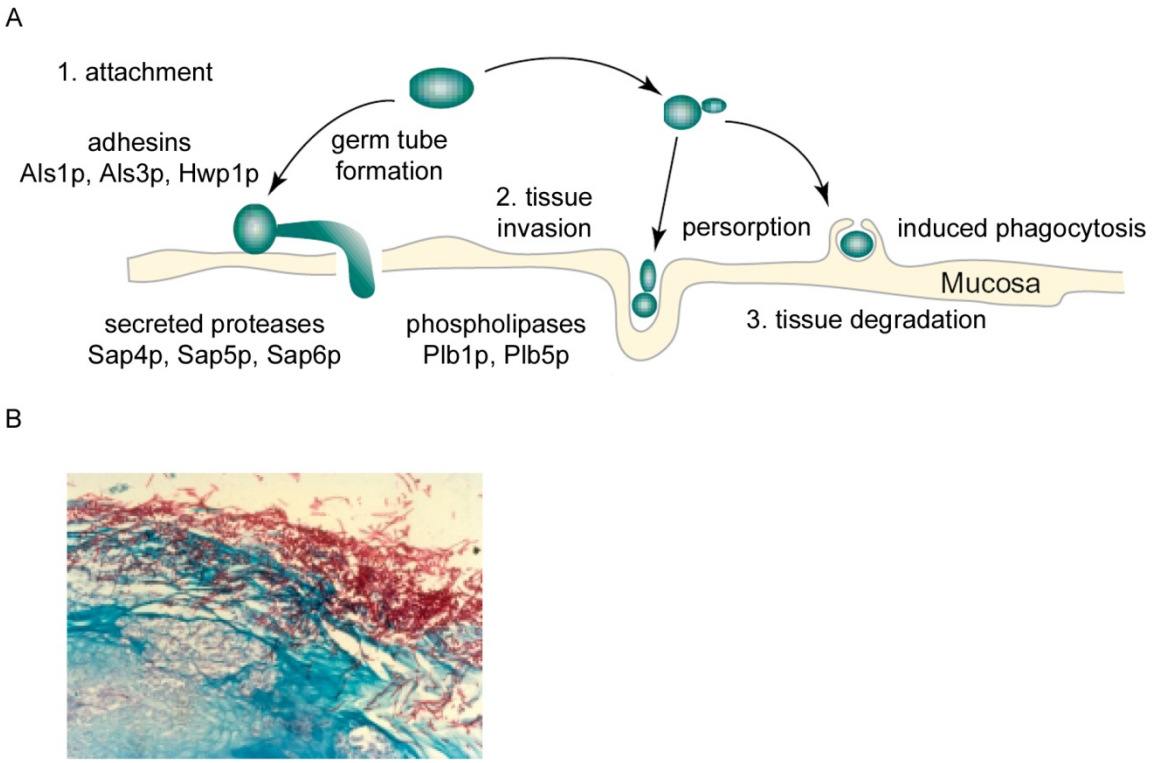

Figure 1. 10 Several virulence factors contribute to early events in the pathogenesis of candidiasis. (A) At the mucosal surface, attachment (1) of *C. albicans* cells to host cells is promoted by adhesins such as Als1p, Als3p, and Hwp1p. Tissue invasion (2) results from *C. albicans* cells forming filaments or being taken in by host cells via persorption or induced phagocytosis. Subsequent tissue degradation (3) is caused by secreted factors (SAPs, PLs), by mechanical force exerted by hyphae or by a combination of both. (B) *C. albicans* yeast and hyphae with predominantly hyphae invading an epithelial surface are shown. Adapted from Calderone & Fonzi (2001); Gow *et al*. (2002).

C. albicans also secretes lipases that catalyze the hydrolysis of ester bonds at the interface between the insoluble triacylglycerol phase and the aqueous phase in which the enzyme is dissolved. Ten genes encoding lipases (*LIP1-10*) have been identified in *C. albicans*. Interestingly, transcripts of most lipase genes were detected during the yeast-to-hypha transition (Hube *et al.*, 2000). *LIP* genes are expressed in a wide range of *Candida*

infections, both *in vitro* and *in vivo*, yet the role and function of lipases in infection remains to be elucidated (Schaller *et al*., 2005).

1.4.1.3 Morphogenesis

The role of hyphae in virulence has been difficult to assess given that most strains defective in filamentation lack transcription factors that regulate both hyphal growth and the expression of virulence genes. These strains are unable to produce hyphae and are often compromised in virulence. However, it is impossible to determine whether reduced virulence stems from the absence of hyphae or from the absence of expression of virulence genes that are co-regulated with hyphal growth. The identification and characterization of the G1 cyclin encoded by *HGC1* solved this conundrum. A hypha-specific gene, *HGC1* is regulated by the cAMP-PKA and Tup1p signaling pathways (Zheng & Wang, 2004). An *hgc1/hgc1* mutant exhibited a severe filamentation defect in various hypha-inducing conditions and was dramatically attenuated in a model of systemic candidiasis, suggesting that *HGC1* is involved in hyphal growth and in virulence. To this date, *HGC1* remains the only HSG required for hyphal development. Strikingly, several HSGs, including *HWP1*, *HYR1*, and *ECE1* were still expressed in an *hgc1/hgc1* mutant despite the fact cells were non filamentous (Zheng & Wang, 2004). These findings demonstrate that virulence is associated to the hyphal form, and does not only stem from the form-specific virulence genes that are expressed.

Additional lines of evidence demonstrate the association between the yeast-to-hypha transition and virulence. First, impairing filamentation using engineered strains in which morphogenesis was manipulated externally attenuated virulence in a model of systemic candidiasis (Carlisle *et al.*, 2009; Saville *et al.*, 2003; Saville *et al.*, 2006). Second, filamentation was necessary for *C. albicans* to evade phagocytes (Lorenz *et al*., 2004), to escape from blood vessels (Phan *et al.*, 2000), and to colonize medical devices by forming biofilms (Nobile & Mitchell, 2005; Nobile *et al.*, 2006b). Third, hyphae are commonly

observed in biopsies of *C. albicans*-infected organs (Odds, 1988a). Thus, morphogenesis contributes to the overall virulence of *C. albicans*.

1.4.2 Hgc1p and the cyclin-dependent kinase Cdc28p

In *C. albicans*, morphogenesis is the transition from growth in a budding yeast form to growth in a hyphal form. Thus, the change in cell shape involves a change in growth patterns. In *S. cerevisiae* and *C. albicans* yeast cells, growth pattern changes (from isotropic to polarized stages) are cell-cycle dependent and controlled by the cyclin-dependent kinase (CDK) Cdk1p/Cdc28p which associates sequentially with G1 and G2 cyclins. In contrast, during hyphal growth, cells commit to continuous polarized growth. Curiously, although hyphal growth is cell cycle-independent, the CDK Cdc28p and various cyclins are involved in morphogenesis (Hazan *et al.*, 2002; Wang, 2009). Homologues of *S. cerevisiae* cyclins have been identified, including G1 cyclins Ccn1p, Hgc1p, and Cln3p and B-type cyclins Clb2p and Clb4p. Ccn1p and Cln3p are required for hyphal growth (Bachewich & Whiteway, 2005; Chapa y Lazo *et al.*, 2005; Loeb *et al.*, 1999), while B-type cyclins Clb2p and Clb4p negatively regulate polarized growth (Bensen *et al.*, 2005).

Compared to other cyclins, Hgc1p is the main cyclin involved in hyphal growth, as it promotes filamentation in response to all hypha-inducing signals (Zheng & Wang, 2004). Unlike other cell-cycle regulated genes, *HGC1* is a gene whose expression is induced only upon the yeast-to-hypha transition, as a result of the activation of the cAMP-PKA signaling pathway and the derepression of the Tup1p-Nrg1p signaling pathway (see sections 1.3.2; 1.3.6). Hgc1p is involved in polarized growth, as it localizes to apical cells of hyphae, where it maintains the localization of actin and polarisome component Spa2p at the hyphal tip. In addition, Hgc1p interacts with Cdc28p, thereby forming the $Cdc28p^{Hgc1p}$ complex (Zheng & Wang, 2004). Recent findings have demonstrated the role of CDKs in hyphal growth by establishing links between $Cdc28p^{Hgc1p}$ and key components of the polarity machinery, including the Cdc42p module (Zheng *et al*., 2007), the transcription factor Efg1p (Wang *et al*., 2009), and the septins Cdc11p (Sinha *et al.*, 2007) and Sep7p

(Clemente-Blanco *et al.*, 2006; Gonzalez-Novo *et al.*, 2008). These findings have been reviewed elegantly (Wang, 2009) and are summarized in Figure 1.11.

Interestingly, some molecular events involved in polarized growth are activated prior to the expression of hypha-specific genes. Indeed, G1 cyclin Ccn1p, present in cells prior to Hgc1p, is required to rapidly establish polarized growth. $Cdc28^{Ccn1p}$ phosphorylates Cdc11p and the Spitzenkörper component Sec2p immediately upon hyphal induction, independently of the MAP kinase and cAMP-PKA signaling pathways (Bishop *et al.*, 2010; Sinha *et al.*, 2007). Concomitantly, morphogenetic signaling pathways are activated, resulting in *HGC1* transcription. Once it reaches sufficient levels, Hgc1p associates with Cdc28p, thereby replacing Ccn1p, which is destined to degradation in the cell cycle. Contrary to Ccn1p, Hgc1p is required to maintain polarized growth. Thus, sustained *HGC1* expression is crucial to hyphal growth, given that the Hgc1p protein is degraded rapidly (Wang *et al*., 2007). Recent findings have shown that the transcription factor Ume6p is required to maintain both the levels and durations of *HGC1* expression in response to hypha-inducing signals (Carlisle & Kadosh, 2010). Together, these findings suggest that the hyphal growth program is initiated at the posttranscriptional level, but is maintained by transcriptional events (Sinha *et al*., 2007).

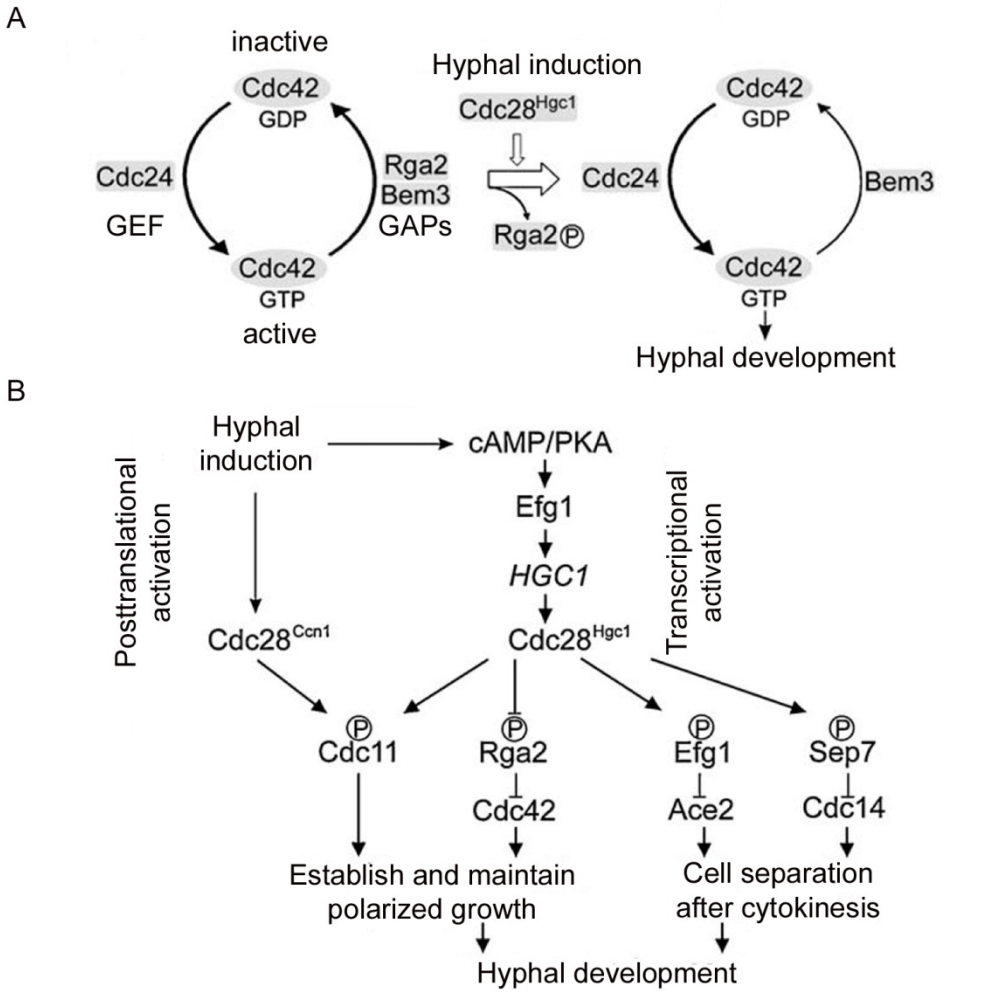

Figure 1. 11 Role of cyclin-dependent kinases in morphogenesis in *C. albicans*.

(A) The Rho-GTPase Cdc42p cycles between a GDP-bound inactive state and a GTP-bound active state. The guanine nucleotide exchange factor (GEF) Cdc24p activates Cdc42p to its GTP-bound form and GTPase-activating proteins (GAPs) Rga2p and Bem3p return it to its GDP-bound inactive form. High levels of Cdc42p-GTP are required to sustain polarized growth during morphogenesis. Upon hyphal induction, $Cdc28p^{Hgc1p}$ inactivates Rga2p by hyperphosphorylation, thereby inhibiting its GAP activity and resulting in a local increase of Cdc42p-GTP. (B) In *C. albicans*, hyphal development depends on two cyclin-dependent kinases (CDKs) which promote germ tube formation and ensure continuous hyphal extension. Upon hyphal induction, $Cdc28^{Ccn1p}$ is activated immediately and phosphorylates Cdc11p, thereby promoting germ tube formation. Meanwhile, activation of the cAMP signaling pathway results in the transcriptional activation of *HGC1*. $Cdc28p^{Hgc1p}$ phosphorylates Cdc11p and Rga2p, thereby maintaining polarized growth. The CDK also phosphorylates Efg1p and Sep7p, thereby preventing cell separation after cytokinesis. Adapted from Wang (2009).

1.5 *Candida* infections

In the genus *Candida*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis* are encountered as gut commensals and as opportunistic pathogens, yet *C. albicans* remains the species most commonly associated with infections (Miceli *et al.*, 2011; Odds *et al.*, 2007). *C. albicans* is a normal inhabitant of human microbiota, colonizing asymptomatically the oral cavity and gastrointestinal tracts of 60 to 90% of healthy adults and the genitourinary tracts of 15 to 25% of healthy women (Fidel, 2002) (Figure 1.12). *C. albicans* is also found on the skin, albeit to a lesser extent (Berman & Sudbery, 2002). Generally, few healthy carriers develop clinical signs of disease. However, when immunological and mechanical defenses are compromised, *C. albicans* may establish an infection, revealing its opportunistic nature. Several factors, including natural, dietary, mechanical, and iatrogenic factors (Table I), may predispose to disease by altering the host environment, enabling *C. albicans* to proliferate, to invade tissues, and to cause an infection, i.e. candidiasis (Odds, 1988a).

Table I Predisposing factors for candidiasis[a]

Types of factors	
Natural	microbial infections, AIDS, endocrine dysfunction (e.g. diabetes), lymphocyte defects, phagocyte abnormalities, pregnancy, infancy, old age
Dietary	excess (e.g. carbohydrate-rich diets), deficiencies (e.g. vitamin deficiencies)
Mechanical	trauma, burns, wounds, occlusion, prosthetic devices (e.g. dentures), thumb sucking
Iatrogenic	antibiotics eliminating the gut microflora, immunosuppressive drugs altering host defenses, surgery, insertion of mechanical devices (e.g. catheters)

[a]adapted from Odds (1988a)

The type of *Candida* infection is dictated by a host's immune status and microbial flora and by the site of carriage (Hube, 2004). *C. albicans* is a successful pathogen as it can proliferate in a multitude of host niches, thereby establishing disease at various body sites. Different forms of oral infections are seen in infants, the elderly, and patients infected with human immunodeficiency virus (HIV). Vulvovaginitis arises mainly in women; infections of the male genitalia are much less common, but still reported. Cutaneous infections with *Candida* are seen on areas of the skin that are moistened by occlusion or maceration. Generally, these superficial forms of candidiasis (section 1.5.1) are easy to diagnose and to treat.

In addition to causing superficial mycoses, *C. albicans* is also the causative agent of invasive candidiasis (see section 1.5.2). When the pathogenic yeast gains access to the bloodstream, often from the gut, but also via catheters and other devices, it can disseminate and infect deep tissues and organs. Such infections, seen in immunocompromised individuals only, are severe, difficult to treat and often lethal. Invasive candidiasis has become increasingly important due to a growing population of immunocompromised and/or hospitalized patients with severe underlying disease (Enoch *et al.*, 2006; Pfaller & Diekema, 2007). Indeed, *C. albicans* is the most common cause of disseminated and invasive fungal infections in hospital settings, with associated mortality rates of 38-49% (Horn *et al.*, 2009; Miceli *et al.*, 2011; Pfaller & Diekema, 2007). Additionally, it is now the third or fourth leading cause of microbial septicemia in hospital settings, having outstripped many bacterial infections in terms of incidence and morbidity (Almirante *et al.*, 2005; Beck-Sague & Jarvis, 1993; Miceli *et al.*, 2011; Tortorano *et al.*, 2004).

1.5.1 Skin and mucosal candidiasis

Candida infections of the skin and mucous membranes include oropharyngeal, esophageal, vulvovaginal, and gastrointestinal candidiasis. Oropharyngeal candidiasis (OPC) involves infections of the hard and soft palate, tongue, buccal mucosa, and floor of

the mouth caused by *C. albicans*. It presents either as white, curd-like lesions (thrush or pseudomembranous candidiasis) or reddened patches (erythematous candidiasis) (Laudenbach & Epstein, 2009). Thrush is characterized by removable white plaques that may be scraped away to reveal a bleeding surface. Symptoms of OPC usually include an alteration of taste, inflammation in the mouth with oral pain, and burning of the mouth. Thrush occurs in infants, the elderly, HIV-infected individuals, and patients afflicted with natural predisposing factors (see Table I) or undergoing broad-spectrum antibiotic therapy (Cannon *et al.*, 1995). Erythematous OPC (a reddened area without removable white spots or plaques) has been reported mainly in patients with HIV infection, but also in patients with denture stomatitis. In addition to thrush and erythematous OPC, hyperplastic candidiasis (*Candida* leukoplakia) and angular cheilitis (perlèche) are two other subtypes of OPC (Ruhnke, 2002). OPC is usually present at the time esophageal candidiasis (EC) is diagnosed. Patients with EC may present with dysphagia (difficulty swallowing) or odynophagia (pain while swallowing) and develop ulcers and erosions on the esophagus (Ruhnke, 2002).

The benign vulvovaginal candidiasis (VVC) or vaginitis is the most common type of *Candida* infection. An estimated 75% of all women experience at least one episode of VVC in their lifetime, while 5 to 10% develop recurrent VVC (Ruhnke, 2002; Sobel, 2002). Candidiasis of the male genitalia can also occur, albeit less commonly than VVC. *C. albicans* is the main causative agent in approximately 85 to 90% of VVC cases, followed by *C. glabrata* and *C. tropicalis* (Fidel & Sobel, 2002). VVC involves infections of the vaginal lumen and the vulva, causing vulvovaginal burning, itching, soreness, an abnormal discharge, and dyspareuria. A diagnosis of VVC is suggested clinically by pruritus (i.e. itching) and erythema in the vulvovaginal area, which may be seen together with white patches that resemble those seen in oral thrush. Factors that predispose to VVC include hormonal perturbations (e.g. oral contraceptive usage, hormone replacement therapy, and pregnancy) and any disturbance of the vaginal flora (e.g. antibiotic or immunosuppressive therapy) (Fidel & Finkel-Jimenez, 2007).

Gastrointestinal (GI) candidiasis is defined as infections of the stomach and the small and large intestines (Fidel & Finkel-Jimenez, 2007). It is caused by the disruption of the normal gut microflora, resulting in the overgrowth of *C. albicans*. Compared to OPC and VVC, GI candidiasis is relatively rare. However, it is an important risk factor for disseminated candidiasis in immunocompromised individuals (see section 1.5.2), including cancer and transplant patients receiving immunosuppressive or antibiotic prophylaxis therapy and HIV-infected individuals (Matthews & Witek-Janusek, 2002).

Candida species, and mostly *C. albicans*, can cause superficial infections in both the skin and the nails (Odds, 1988b). The pathogenic yeast has a predilection for warm and moist environments, such as the skin folds under the breasts and gluteus and the bending creases between the fingers and toes, the armpits and groin, created by maceration and occlusion (Lopez-Martinez, 2010). Cutaneous candidiasis presents as erythematous rashes causing eruption, secretions, itching, and burning. *Candida* skin infection is a common cause of diaper rash in infants, but can also occur in the elderly, and in obese adult patients (Ruhnke, 2002).

1.5.2 Invasive candidiasis

Invasive *Candida* infections include candidemia (infection of the blood), hematogenously disseminated candidiasis, and deep-seated candidiasis of one or more organs (Filler & Kullberg, 2002; Kullberg & Filler, 2002). Invasive candidiasis stems from *C. albicans* cells being translocated from the GI tract or from the skin into the bloodstream (Kullberg & Filler, 2002) (Figure 1.12). Several factors promote translocation, including disruption of cutaneous barriers (catheters, burns, surgical wounds, and trauma) and mucosal barriers (surgery, chemotherapy-related mucositis, GI malignancy), GI candidiasis (antibiotic therapy), and impaired cell-mediated immunity (immunosuppressive therapy, neutropenia) (Sims *et al.*, 2005). Deep-seated candidal infections of organs may arise from hematogenously disseminated candidiasis or from the direct inoculation of an organ by a

contaminated catheter or other medical devices (Kojic & Darouiche, 2004; Sims *et al.*, 2005). Although there are no specific signs that characterize candidemia or hematogenously

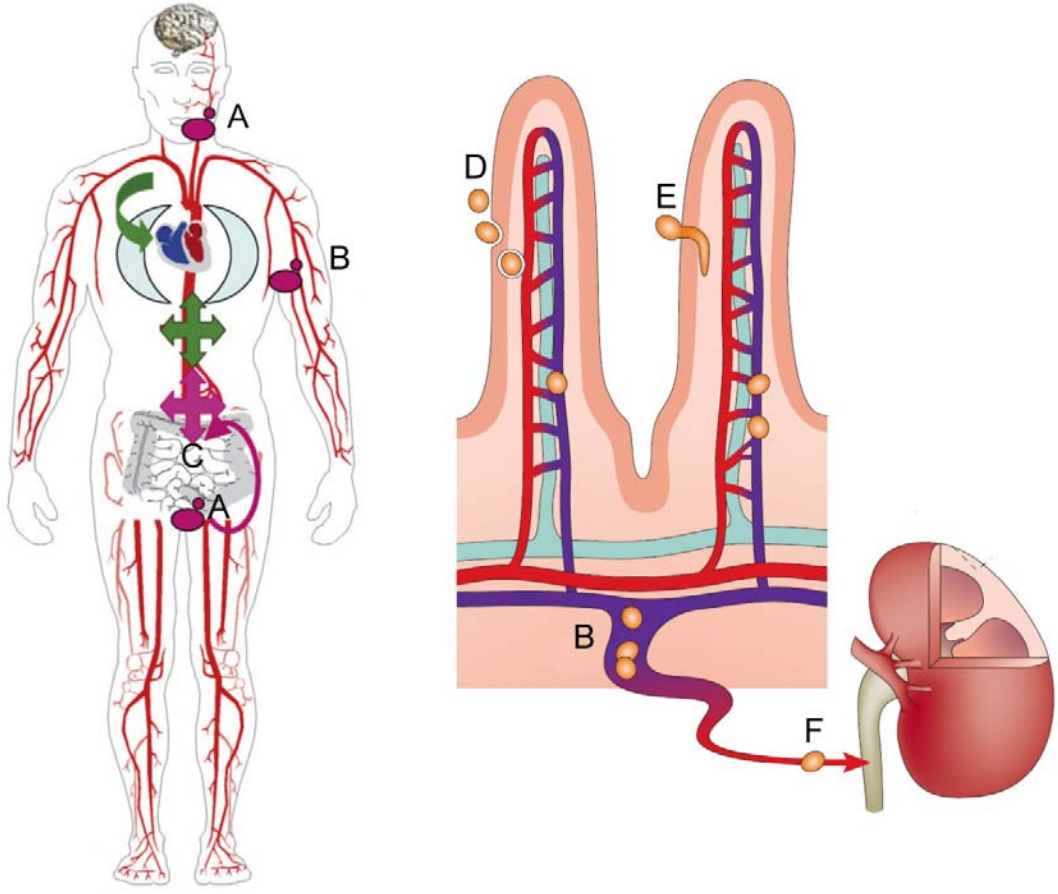

Figure 1. 12 Infection cycles of *Candida albicans*.

C. albicans is a normal resident of the skin, oral cavity, genitourinary, and gastrointestinal tracts. Harmless in the commensal state, *C. albicans* is an opportunistic pathogen, causing infection when immunological and mechanical host defenses are compromised. *Candida* infections of the skin and mucous membranes occur in both healthy and immunocompromised individuals (A), while invasive candidiasis is only seen in severely immunocompromised individuals (B, F). Invasive candidiasis (candidemia, hematogenously disseminated candidiasis, and deep-seated candidal infections of organs) generally stems from the translocation of *C. albicans* cells from the gastrointestinal mucosa into the bloodstream (B, C). *C. albicans* enters epithelial microvilli through persorption of yeast cells (D) or germination (E). After gaining access to the bloodstream, yeast cells are hematogenously disseminated (B) and can infect host tissues such as the kidney (F). Adapted from Roman *et al*. (2007); Chauhan *et al*. (2006).

disseminated candidiasis, these infections usually present as a persistent fever in a seriously ill patient despite antibacterial treatment. Some patients present with the sepsis syndrome, including fever, hypotension, tachycardia, and tachypnea (Kullberg & Filler, 2002). Since the diagnosis of invasive candidiasis is difficult to establish, antifungal therapy is often initiated on the presumption of this type of infection.

In invasive candidiasis, *C. albicans* cells first enter the bloodstream, either by persorption or germination of cells across epithelial cells, by seeding from contaminated devices, by invasion of epithelially denuded surfaces, or via trauma or surgically related inoculation (Bendel *et al.*, 2003; Cole *et al.*, 1996; Hawser & Douglas, 1994). To invade tissues, cells exit the circulation by adhering to the endothelial lining of blood vessels and transmigrating across the endothelium (Figure 1.12) (Grubb *et al.*, 2008). Two theories explain adhesion and transmigration processes of *C. albicans* cells. One theory proposes that yeast cells must first undergo the morphogenetic transition in order for hyphal cells to bind to and damage the endothelial lining of blood vessels before transmigrating from the bloodstream into tissues (Phan *et al.*, 2000; Sanchez *et al.*, 2004). Conversely, the second theory suggests that the yeast-to-hypha transition is not a prerequisite for yeast cells to extravasate from blood vessels (Bendel *et al.*, 2003; Saville *et al.*, 2003).

Adhesion of fungal cells to endothelial cells is mediated by *C. albicans* cell wall components, including proteins (integrin-like adhesins and the *ALS* gene products) and carbohydrates (e.g. mannans) (Grubb *et al.*, 2008). Integrin-like adhesins $\alpha_M\beta_2$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ mediate adhesion of yeast and/or hyphal cells to human endothelial cells by binding cell adhesion molecules (CAM) (Gustafson *et al.*, 1991; Santoni *et al.*, 2001; Spreghini *et al.*, 1999). For instance, $\alpha_M\beta_2$ binds to ICAM-1 and -2, $\alpha_v\beta_3$ interacts with PECAM-1, while $\alpha_v\beta5$ lacks a known ligand. The large GPI-linked cell surface glycoproteins encoded by the *ALS* genes (discussed in 1.4.1.1) have contrasting roles in adhesion and transmigration. Among the eight members of the Als family, Als1p, Als2p, Als3p, Als4p and Als9p are required for adhesion to endothelial cells. To this date, N-cadherin, which binds the hypha-

specific Als3p, is the only ligand for the *ALS* gene products found on endothelial cells (Phan *et al.*, 2007). Several sugar residues, found on yeast and hyphal cells, are recognized and bound by host pattern recognition receptors. For instance, N- and O-linked mannosyl residues are bound by the mannose receptor and Toll-like receptor 4 (TLR-4), respectively (Netea *et al.*, 2006). Similarly, phospholipomannan and β-mannosides are recognized and bound by TLR-2 and galectin-3 (Jouault *et al.*, 2003; Jouault *et al.*, 2006).

Upon adhering to endothelial cells, *C. albicans* cells migrate across the endothelial barrier. Several mechanisms have been proposed to explain this process. One model proposes that hyphae bind to N-cadherin at the surface of endothelial cells via the hypha-specific protein Als3p, thus promoting their endocytosis and passage through the endothelial cell layer (Filler, 2006; Phan *et al.*, 2005; Phan *et al.*, 2007). Endothelial endocytosis is not restricted to hyphae, as adherent yeast cells are also endocytosed (Phan *et al.*, 2000). Another model involves the extension and penetration of hyphae through endothelial cells, destroying them in the process. Alternatively, adherent *C. albicans* cells may pass between adjacent endothelial cells by cyclical switching of adhesion molecules (Grubb *et al.*, 2008).

1.6 Treatment of candidiasis

Candida infections are treated with antifungal drugs. Currently, there are four main classes of antifungal agents which perturb essential processes involved in cell wall biogenesis and in ergosterol and DNA synthesis (Odds *et al.*, 2003) (discussed in section 1.6.1). While superficial mycoses remain fairly easy to treat with conventional antifungal drugs, invasive *Candida* infections pose a greater challenge. Indeed, treatment of such infections is complicated by the limited arsenal of antifungal drugs, the severe side effects in patients, the development of antifungal drug resistance, and the emergence of species refractory to conventionally used agents (Anderson, 2005; Cowen *et al.*, 2002; Sanglard &

Odds, 2002). Additionally, invasive *Candida* infections are on the rise due to a growing population of immunocompromised and/or hospitalized patients with severe underlying disease (Enoch *et al.*, 2006). Combined, these factors underscore the need for new targets or new strategies in antifungal therapy (Calugi *et al.*, 2011; Espinel-Ingroff, 2009).

1.6.1 Classical antifungal drugs

Conventional antifungal drugs target different cellular processes that are essential for growth. According to their targets and modes of action, antifungal drugs can be classified in four categories, including polyenes, nucleic acid inhibitors, ergosterol biosynthesis inhibitors, and echinocandins (Figure 1.13A). The emergence of resistance is an important aspect of antifungal therapy. The mechanisms developed by *C. albicans* to resist to conventional antifungal agents have been reviewed elsewhere (Sanglard & Bille, 2002; Sanglard & Odds, 2002; Sanglard & White, 2007; White *et al.*, 1998) and are summarized in Figure 1.13B.

1.6.1.1 Polyenes

Discovered in the early 1950s, polyenes such as amphotericin B (AmB) and nystatin are heterocyclic amphipathic molecules that insert into membranes, bind to ergosterol, and aggregate to form pores. These pores disrupt the integrity of the plasma membrane, allowing small molecules to diffuse across it. Altered permeability and leakage of vital cytoplasmic components result in cell death (Sanglard & Bille, 2002). A natural antibiotic produced by *Streptomyces nodosus*, AmB has a fungicidal activity against *Candida* species as well as a broad spectrum of activity against other fungi (Ellis, 2002). Moreover, resistance to AmB is rare, given that it is fungicidal. AmB is used to treat severe, life-threatening systemic infections caused by *C. albicans* or by *Candida* species that are intrinsically azole-resistant (e.g. *C. glabrata* and *C. krusei*) or that have acquired resistance to azoles (Rex *et al.*, 2000).

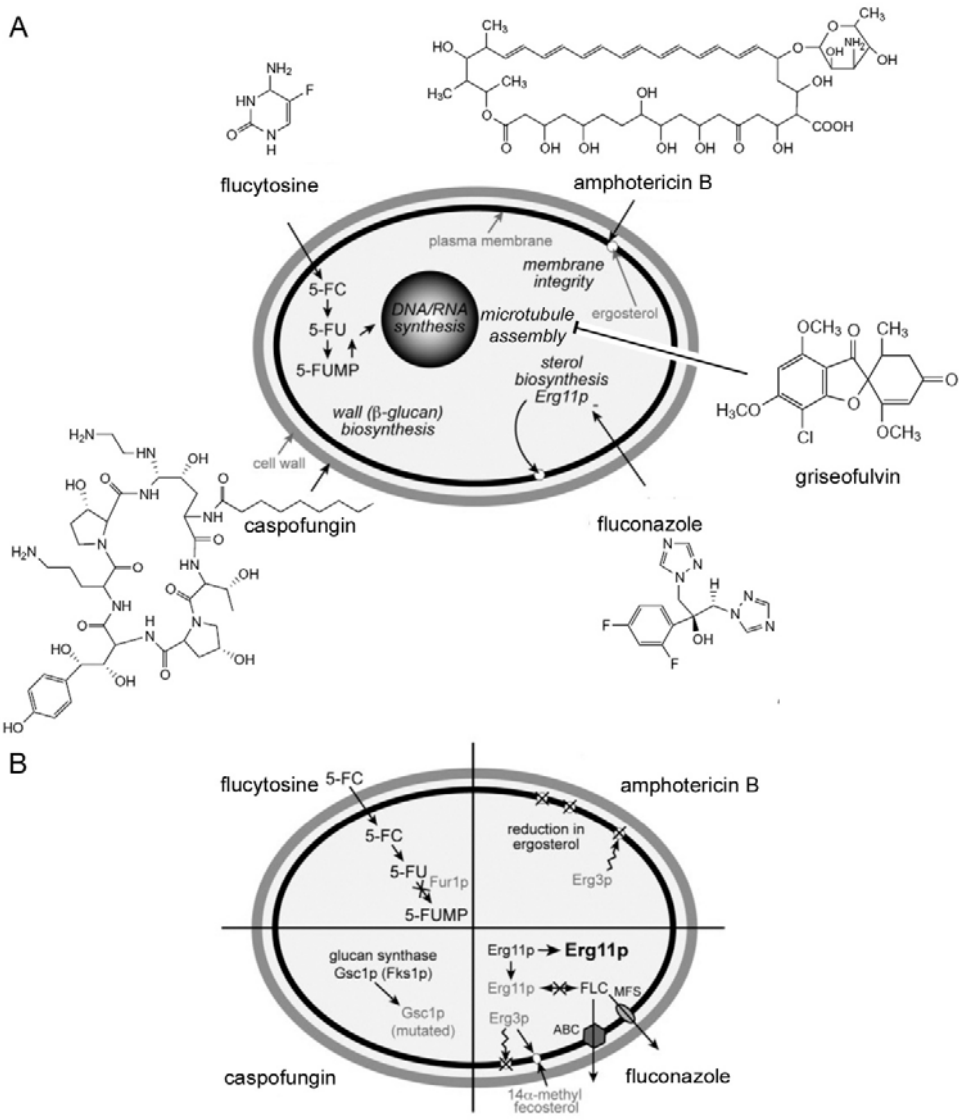

Figure 1. 13 Antifungal drug targets and resistance mechanisms.

(A) 5-FC targets DNA and RNA synthesis. Polyenes such as amphotericin B and nystatin bind to plasma membrane ergosterol, creating pores which lead to efflux of cations and cell death. Azoles inhibit the product of *ERG11* that catalyzes the 14α-demethylation of lanosterol, resulting in the accumulation of toxic methylated sterols in the plasma membrane and the depletion of ergosterol. Echinocandins inhibit synthesis of β-1,3 glucan polysaccharides, thereby affecting cell wall and cell membrane composition and integrity. Griseofulvin interferes with microtubule assembly and inhibits mitosis. (B) Summary of resistance mechanisms. (see text for details). Adapted from Odds *et al*. (2003); Cannon *et al*. (2007).

Although polyenes have a greater affinity for ergosterol-containing membranes than for cholesterol-containing membranes, they can still bind the main sterol present in host cell membranes, which accounts for their toxic potential in humans. In addition, polyenes are insoluble in aqueous solutions, and have to be complexed with other agents for clinical administration. For instance, complexing AmB to sodium deoxycholate increased solubility, yet such formulations were quite nephrotoxic. Lipid formulations of AmB have since been developed to circumvent solubility and toxicity issues, and are generally better tolerated by patients (Laniado-Laborin & Cabrales-Vargas, 2009).

1.6.1.2 5-Flucytosine

5-Flucytosine (5-FC) belongs to the class of pyrimidine analogues that were developed in the 1950s as anticancer agents. 5-FC is taken up by a cytosine permease (Fcy1p and Fcy2p) and is deaminated by a cytosine deaminase (Fca1p) and converted to 5-fluorouracil (5-FU), a potent antimetabolite (Sanglard & Bille, 2002). Incorporated into RNA, 5-FU causes premature chain termination, but it also inhibits DNA synthesis by affecting thymidylate synthase (Sanglard & White, 2007). 5-FC has a fungicidal activity, as it inhibits nucleic acid synthesis and division. However, its spectrum of activity is restricted to fungi that harbor the enzymes required to convert it into 5-FU, namely *Candida* species and *Cryptococcus neoformans*. Additionally, 5-FC is usually administered in combination with AmB because resistance occurs at a high frequency. Resistance to 5-FC may be due to a defective cytosine permease, a deficiency or lack of enzymes involved in the metabolism of 5-FC, or a deregulation of the pyrimidine biosynthesis pathway (Sanglard & Bille, 2002).

1.6.1.3 Ergosterol biosynthesis inhibitors

Ergosterol biosynthesis inhibitors (EBIs) form a diverse group of antifungals which includes azoles, allyamines, thiocarbamates, and morpholines. These agents inhibit the biosynthesis of ergosterol, the main sterol of fungal membranes, resulting in its depletion

and replacement with unusual sterols. Altered membrane permeability and fluidity affect membrane-bound enzymes, resulting in the inhibition of cell growth. The ergosterol pathway is divided into an early pathway (producing squalene from acetate) and a late pathway (producing ergosterol from squalene). EBIs inhibit steps in the late pathway. For instance, allyamines (e.g. terbinafine) and thiocarbamates inhibit squalene epoxidase (product of *ERG1*) with fungicidal consequences in susceptible species. These include many filamentous fungi, but very few pathogenic yeasts. Topical or oral preparations of terbinafine are used to treat skin and nail infections (onychomycosis) caused by dermatophytes. It is usually well tolerated, but may cause gastrointestinal upset, taste disturbance, and transient elevation of liver enzymes (Chen & Sorrell, 2007). Morpholines (e.g. amorolfine) inhibit the products of *ERG2* and *ERG4*, resulting in the formation of ignosterol which blocks cell growth. Amorolfine can only be used for topical treatment of superficial mycoses such as VVC (Sanglard & Bille, 2002).

Azoles are the most important EBIs and are regrouped into two classes: imidazoles (ketoconazole, miconazole, and clotrimazole) and triazoles (fluconazole, voriconazole, and itraconazole), which have two and three nitrogen atoms in their azole ring, respectively. Azoles target the cytochrome P450 enzyme, 14α-demethylase, encoded by *ERG11*. Erg11p, which contains a protoporphyrin ring at its active site, catalyzes the 14α-demethylation of lanosterol. Azoles inhibit Erg11p's enzymatic activity by binding to its iron atom via a nitrogen atom contained in the imidazole or triazole ring, thereby interfering with the conversion of lanosterol to ergosterol and resulting in the accumulation of methylated sterols in the plasma membrane (Odds *et al.*, 2003). Azoles inhibit cell growth, but do not kill cells, and thus have a fungistatic rather than a fungicidal activity. Yet, they have a broad spectrum of activity and are less toxic than AmB, which is why they are used to treat various types of *Candida* infections. For instance, fluconazole is used to treat mucosal candidiasis (VVC, OPC) and chronic mucocutaneous candidiasis. It is also the drug of choice to treat invasive candidiasis. Itraconazole is effective to treat fluconazole-resistant superficial candidiasis.

The fungistatic properties of azoles have played a role in the emergence of resistance. Indeed, static drugs can leave large populations of survivor cells that are exposed to strong selection for resistance (Anderson, 2005). These cells accumulate mutations which enable them to grow in the presence of the antifungal drug. Various mechanisms account for the development of resistance to azoles (Figure 1.13B), including enhanced drug efflux mediated by multidrug transporters (Cdr1p, Cdr2p, and Mdr1p) (Sanglard *et al.*, 1995; Sanglard *et al.*, 1997; Wirsching *et al.*, 2000), decreased affinity of azoles for Erg11p caused by mutations (Marichal *et al.*, 1999), overexpression of *ERG11* (Perea *et al.*, 2001), and alteration of the ergosterol pathway caused by the absence of Erg3p (Nolte *et al.*, 1997).

1.6.1.4 Echinocandins

Recently developed, echinocandins are fungal secondary metabolites comprised of a hexapeptide core with a lipid side-chain responsible for antifungal activity. Echinocandins target β-1,3 glucan synthase, which is part of an enzyme complex involved in the synthesis of cell wall β-1,3 glucan polysaccharides (Sanglard & Bille, 2002). Although the mechanistic details of the inhibition of glucan synthesis by echinocandins is still obscure, it appears that the compounds bind to Fsk1p, a subunit of β-1,3 glucan synthase. Disruption of the cell wall structure leads to osmotic instability and ultimately to lysis of the cell (Odds *et al.*, 2003). Echinocandins include caspofungin, anidulafungin, and micafungin, which differ in their lipid side-chains. While the latter two compounds are still in clinical trial, caspofungin has been approved for the treatment of *Candida* infections (Sanglard & White, 2007). So far, echinocandin resistance has only been reported in *C. albicans* strains that have point mutations in Gsc1p, a subunit of β-1,3 glucan synthase (Baixench *et al.*, 2007). Moreover, echinocandins appear to be well tolerated, given that they affect a fungi-specific target (i.e. fungal cell wall).

1.6.1.5 Griseofulvin

While griseofulvin was the first antifungal drug to be developed, its mechanism of action is still unknown. It appears that griseofulvin interferes with microtubule assembly and inhibits mitosis (Odds *et al*., 2003). Griseofulvin has a narrow spectrum of activity, and is used to treat superficial mycoses caused by dermatophyte fungi, such as ringworm and athlete's foot.

1.6.2 Targeting virulence: a new paradigm for antifungals

Antifungals exert either fungicidal or fungistatic activities towards *C. albicans* by interfering with processes that are essential for its growth (see section 1.6.1). Intensive prophylactic and therapeutic uses of antifungals have selected for drug-resistant strains (Anderson, 2005; Cowen & Steinbach, 2008; Sanglard & White, 2007). One way to preclude resistance is to develop a wider repertoire of antifungal drugs whose targets differ from those of conventional drugs. Interestingly, targeting virulence rather than essential processes has been postulated as a new paradigm for the development of antifungal agents, following the successful development of drugs targeting bacterial virulence in antimicrobial therapy (Alksne & Projan, 2000; Calugi *et al.*, 2011; Gauwerky *et al.*, 2009). Thus, instead of being killed, a pathogen is maintained in a harmless form by hindering its virulence attributes which contribute to pathogenicity. The rationale for the development of drugs targeting virulence instead of growth is that resistance to such agents is less likely to occur, given that selective pressure is reduced on non-essential targets that are only required to colonize host environments (Jiang *et al.*, 2002).

In *C. albicans*, virulence factors are "attributes whose absence in the fungus eliminates or reduces (…) damage in a defined host system in vivo" (Odds *et al.*, 2007) (discussed in section 1.4.1). Virulence factors that are eligible as targets for the development of new antifungal drugs have been reviewed recently, and include secreted aspartic proteases (SAPs), phospholipases, calcineurin, inositol phosphoryl ceramide

synthase (Ipc1p), elastase, and hyphal formation (Calugi *et al.*, 2011; Gauwerky *et al.*, 2009). Several molecules have been shown to be effective against some of these virulence factors. For instance, pepstatin A, saquinavir, and indinavir are peptidomimetic SAP inhibitors, which reduced proteinase activity and attenuated virulence in a model of mucosal infection (Korting *et al.*, 1999). Antibodies developed against virulence factors of *C. albicans*, including a 65-kDa mannoprotein and Sap2p, inhibited the adherence of *C. albicans* to epithelial cells and protected against VVC (De Bernardis *et al.*, 2007). Two immunosuppressive agents, tacrolimus (FK106) and cyclosporin A, which target the virulence factor calcineurin, enhanced the efficacy of antifungal drugs such as fluconazole (Cruz *et al.*, 2002; Sanglard *et al.*, 2003; Steinbach *et al.*, 2007). While these experimental findings are promising, the clinical potential of most of these agents has yet to be demonstrated.

1.6.3 Morphogenesis, a target for the development of novel antifungals

Although recent findings have demonstrated that filamentation is not always required for virulence in systemic candidiasis, morphogenesis still belongs to the realm of *C. albicans* virulence factors (Noble *et al*., 2010). Several lines of evidence underscore the association between the morphogenetic transition and virulence (discussed in 1.4.1.3). Indeed, the ability to switch between different morphological forms is required for virulence in systemic candidiasis (Lo *et al.*, 1997; Saville *et al.*, 2003; Zheng & Wang, 2004), as well as for cells to evade phagocytes (Lorenz *et al*., 2004), to escape from blood vessels (Phan *et al.*, 2000), and to colonize medical devices by forming biofilms (Nobile & Mitchell, 2005; Nobile *et al.*, 2006b).

Because morphogenesis contributes to virulence in *C. albicans*, it may constitute a target for the development of antifungal drugs. Indeed, impairing morphogenesis has been shown to be a means to treat systemic candidiasis. Using engineered strains in which the yeast-to-hypha transition was manipulated externally, the overexpression of *NRG1*, the

repressor of filamentation, inhibited hyphal growth and attenuated virulence in a model of systemic candidiasis (Saville *et al.*, 2003; Saville *et al.*, 2006).

In parallel, molecules have been reported to modulate morphogenesis in *C. albicans*, and may harbor antifungal properties (reviewed in Chapter 3). Among these molecules, fatty acids such as butyric, capric, and undecylenic acids were shown to inhibit germ tube formation in *C. albicans* (McLain *et al.*, 2000; Murzyn *et al.*, 2010; Noverr & Huffnagle, 2004). Likewise, a fatty acid-enriched whey fraction also inhibited the yeast-to-hypha transition (Clement *et al.*, 2007). The hypha-inhibiting properties of whey were attributed to lauric, myristoleic, conjugated linoleic and arachidonic acids. The effect of conjugated linoleic acid (CLA) on hyphal growth in *C. albicans* was further characterized and its mode of action was addressed (see Chapter 2).

1.7 Ras1p, a membrane-associated protein

1.7.1 Brief overview of plasma membrane constituents

The structure and organization of *C. albicans* cells resemble that of other eukaryotic cells. Except for their fungi-specific cell wall, yeast cells have a plasma membrane that forms a barrier surrounding the cytosol which contains intracellular organelles such as ribosomes, double-membraned mitochondria and nuclei, glycogen granules and vacuoles (Odds, 1988a). Approximately 7.5 nm wide, the plasma membrane is a lipid bilayer which contains a mixture of lipids and proteins, held together mainly by noncovalent interactions. The plasma membrane is a dynamic and fluid structure, as its lipid molecules are arranged as a continuous double layer dotted with proteins that can move laterally and rotate on themselves (van der Rest *et al.*, 1995).

Membrane lipids, which vary in size and composition, include phospholipids, sterols (ergosterol), and glycolipids (van der Rest *et al*., 1995). Phospholipids consist of two fatty acyl chains ester-linked to glycerol-3-phosphate. Differences in the length and

saturation of the fatty acyl chains influence the ability of phospholipid molecules to pack against one another, thereby affecting membrane fluidity (discussed below). Ergosterol, a compact and rigid hydrophobic molecule with a polar hydroxyl group, determines membrane rigidity. Ergosterol is also involved in the formation of lipid rafts, which are sterol- and sphingolipid-enriched microdomains (Lingwood & Simons, 2010). As for glycolipids, they are sugar-containing lipid molecules, mostly found on the outer leaflet of the lipid bilayer.

Fatty acids, being constituents of phospholipids, influence the structure of the membrane. For instance, an increase in the ratio of unsaturated to saturated fatty acids increases membrane fluidity. This is caused by the kinks created by the double bonds present in unsaturated fatty acyl chains which decrease the packing ability of phospholipids, thereby increasing membrane fluidity. In contrast, packing increases with increasing length of acyl chains and decreasing extent of unsaturation, resulting in a more ordered structure with decreased fluidity (Alberts *et al.*, 2002). In yeast, fatty acids can be synthesized de novo or taken up from the exterior (Black & DiRusso, 2007). Moreover, exogenous fatty acids can be incorporated into membrane phospholipids, and can modulate the fatty acid profile of phospholipids (Ells *et al.*, 2009; Petschnigg *et al.*, 2009).

Plasma membrane proteins include transport proteins, receptor proteins, and signaling proteins involved in sensing and signaling, selective uptake and/or secretion of solutes across the membrane, and cell wall biogenesis and maintenance (Delom *et al.*, 2006). There are two types of membrane proteins: integral or transmembrane proteins and peripheral proteins. Integral membrane proteins span the membrane through one or more transmembrane domains which interact strongly with the membrane. In contrast, peripheral membrane proteins interact with the membrane, but do not transverse it. These proteins can associate with the membrane indirectly by interacting with integral membrane proteins, or directly by interacting with membrane phospholipids through anchor groups. Anchor groups include prenyl, farnesyl, and geranylgeranyl groups (hydrocarbon moiety),

myristoyl and palmitoyl groups (fatty acyl), and glycosylphosphatidylinositol (GPI) anchors (Alberts *et al.*, 2002). Such lipid modifications enable cytosolic precursors to associate with specific membranes and ensure their proper targeting and subcellular localization.

1.7.2 Ras membrane attachment and subcellular trafficking

Ras proteins are membrane-associated proteins. Proteomic analyses have shown that the *C. albicans* Ras homologue (Ras1p) is a member of the plasma membrane proteome (Alvarez & Konopka, 2007; Cabezon *et al.*, 2009). Synthesized in the cytosol, Ras proteins undergo posttranslational modifications by lipids, which dictate their interactions with distinct membrane compartments and their subcellular localizations. Like the majority of Ras proteins, *C. albicans* Ras1p terminates with the C-terminal CAAX lipid-conjugation motif required for membrane anchorage (Zhu *et al*., 2009). CAAX processing of mammalian Ras proteins is sequential (Figure 1.14). First, farnesyl transferase, a cytosolic enzyme, attaches a farnesyl group to the cysteine residue of the CAAX motif, constituting the initial prenylation (or farnesylation) modification. Next, the farnesylated CAAX sequence targets Ras to the cytosolic surface of the endoplasmic reticulum (ER), where the terminal three amino acids (AAX) are removed proteolytically and the C-terminal farnesylated-cysteine is methylated. Ras is then palmitoylated by a protein acyl transferase (palmitoyl transferase) that attaches palmitic acid via a thioester linkage to the thiol (-SH) group of the cysteine residue located immediately upstream of the farnesylated cysteine. Once palmitoylated, Ras enters the exocytic pathway, trafficking through the Golgi to the plasma membrane (Hancock, 2003). Farnesyl and palmitoyl anchor groups enable Ras to interact with membrane phospholipids and to associate stably to the plasma membrane.

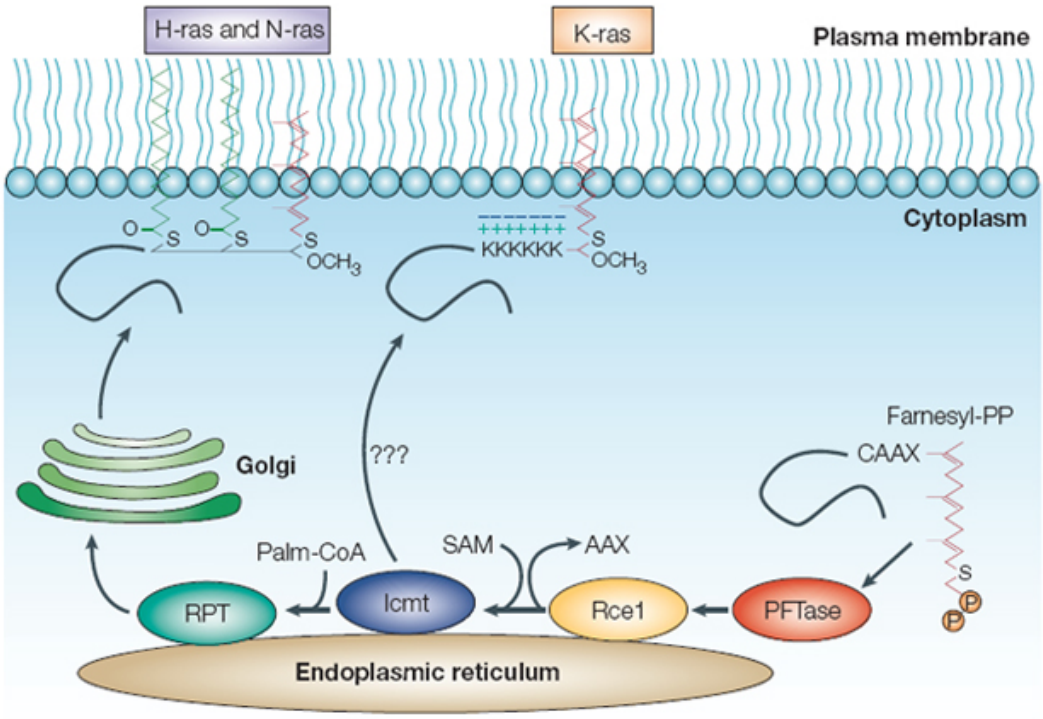

Figure 1. 14 Membrane trafficking of mammalian Ras proteins.

Mammalian Ras proteins undergo sequential posttranslational modifications. First, farnesyl transferase (PFTase), a cytosolic enzyme, attaches a farnesyl group to the cysteine residue of the CAAX motif, constituting the initial prenylation (or farnesylation) modification. Second, the farnesylated CAAX sequence targets Ras to the cytosolic surface of the endoplasmic reticulum (ER), where the terminal three amino acids (AAX) are removed proteolytically and the C-terminal farnesylated-cysteine is methylated with S-adenosylmethionine (SAM). At that point, K-Ras exits the ER and traffics to the plasma membrane through a poorly characterized pathway that bypasses the Golgi. As for H-Ras and N-Ras, they are palmitoylated by an ER-localized palmitoyl transferase (RPT), which attaches palmitoyl CoA (Palm-CoA) via a thioester linkage to the thiol (-SH) group of the cysteine residue located immediately upstream of the farnesylated cysteine. Once palmitoylated, H-Ras and N-Ras enter the classic secretory pathway, trafficking through the Golgi to the plasma membrane. Adapted from Hancock (2003).

Besides being important for the subcellular localization of Ras, another role has been uncovered for palmitoylation (Rocks *et al.*, 2005). Two mammalian Ras isoforms, H- and N-Ras, undergo dynamic cycles of depalmitoylation/palmitoylation, which are accompanied by changes in their subcellular localization between the plasma membrane and the ER/Golgi (Figure 1.15). Palmitoylated Ras accumulates at the plasma membrane, where it is depalmitoylated and recycled to the ER/Golgi. Depalmitoylated Ras is then palmitoylated again at the ER/Golgi and targeted back to the membrane. This continuous cycle maintains Ras in specific intracellular compartments, enabling Ras signaling to be fine-tuned, given that its subcellular location modulates its signal output (Hancock, 2003; Omerovic *et al.*, 2007; Rocks *et al.*, 2005). However, it is not known whether or not the Ras depalmitoylation/palmitoylation cycle operates in *S. cerevisiae*. Moreover, trafficking from the ER to the plasma membrane of yeast Ras appears to depend on functional mitochondria, rather than on a functional secretory pathway (Linder & Deschenes, 2007; Wang & Deschenes, 2006).

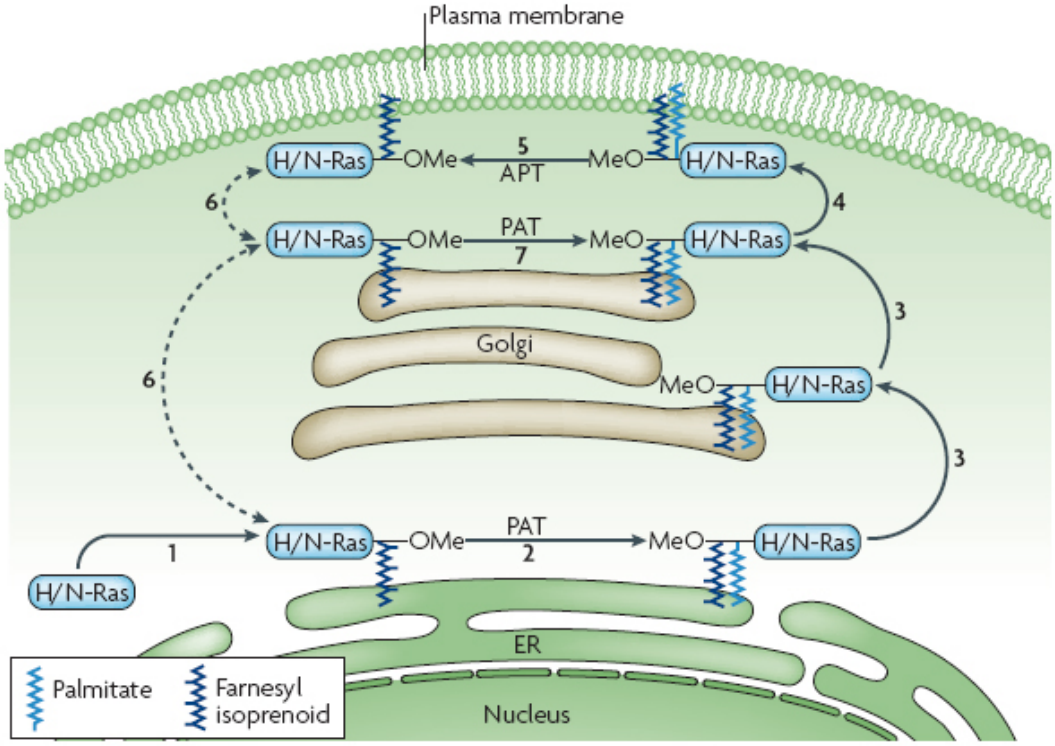

Figure 1. 15 Depalmitoylation/palmitoylation cycle model of subcellular trafficking of H-Ras and N-Ras.

Farnesylation of the CAAX sequence targets H-Ras and N-Ras (H/N-Ras) to the endoplasmic reticulum (ER). (2) Farnesylated H/N-Ras become palmitoylated by a protein acyltransferase (PAT), also known as palmitoyl transferase. Dual-lipid-modified H/N-Ras can then traffic to the Golgi (3) and plasma membrane (4) via the classic secretory pathway. (5) At the plasma membrane, an acylprotein thioesterase (APT) removes the palmitoyl group, allowing farnesylated H/N-Ras to be recycled to the ER/Golgi (6). (7) Another PAT might also repalmitoylate farnesylated H/N-Ras on the Golgi membrane. The depalmitoylation/palmitoylation cycle accounts for the dynamic subcellular trafficking of Ras. Adapted from Linder & Deschesnes (2007).

1.8 Rationale, hypotheses, and research objectives

In *Candida albicans*, morphogenesis is induced by a variety of environmental cues which activate a complex web of signaling pathways composed of transducers, kinases, and transcription factors (discussed in sections 1.2 and 1.3). The yeast-to-hypha transition is a virulence factor of the pathogenic yeast (discussed in 1.4.1.3). A corollary to this idea is that morphogenesis may constitute a target for the development of new antifungal agents. Two reasons support this rationale. First, proof-of-concept experiments using engineered strains in which the yeast-to-hypha transition was manipulated externally demonstrated that harnessing morphogenesis was a means to treat systemic candidiasis (Saville *et al.*, 2003; Saville *et al.*, 2006). Second, targeting virulence processes instead of essential processes is a new paradigm for antifungal drug development (discussed in 1.6.2). Agents that target virulence factors are believed to reduce selective pressure exerted on pathogens and limit the emergence of resistance (Jiang *et al.*, 2002). Concurrently, several small molecules have been reported to modulate morphogenesis (reviewed in Chapter 3). Among these molecules, a fatty acid-enriched whey fraction inhibited the yeast-to-hypha transition in *C. albicans*. Its hypha-inhibiting properties were attributed to several fatty acids, including conjugated linoleic acid (Clement *et al.*, 2007). By inhibiting hyphal growth, conjugated linoleic acid (CLA) interferes with an important attribute of pathogenesis of *C. albicans*, and may harbor therapeutic properties. However, before assessing its therapeutic potential in a clinical context, it is mandatory to address CLA's mechanism of action. For instance, CLA may inhibit the yeast-to-hypha transition by targeting a signaling pathway and/or a regulator involved in morphogenesis (discussed in section 1.3).

The objectives of this work were to characterize the hypha-inhibiting effects of CLA, to identify potential targets mediating these effects, and to elucidate the mechanism by which CLA inhibits the yeast-to-hypha transition in *C. albicans*. These objectives were addressed using a combination of methods. A genomic approach using transcriptional profiling experiments was chosen to gain insight into the transcriptional response of cells to CLA on a genome-wide scale. A phenotypic screen of *C. albicans* mutant strains deleted

for potential CLA targets was also performed to eliminate secondary/indirect CLA targets. Quantitative Northern blot and/or quantitative PCR analyses were conducted to examine the kinetics of expression of several genes. Western blotting techniques were used to examine Ras1p cellular levels, while fluorescence microscopy was used to study the protein's subcellular localization.

By shedding light on CLA's cellular target and its mode of action, it should be possible to predict whether or not the fatty acid could cause deleterious effects in humans, given that the level of conservation between human and yeast is high. In the long run, this work may constitute the rationale for a clinical study examining CLA's therapeutic potential in the context of a *Candida* infection. In addition, this study should enable us to revisit the roles of signaling pathways and morphogenetic regulators in hyphal growth and to expose new findings pertaining to the wiring of these pathways.

2. Chapter 2. Conjugated linoleic acid inhibits hyphal growth in *Candida albicans* by modulating Ras1p cellular levels and downregulating *TEC1* expression

A fatty acid-enriched whey fraction inhibited the yeast-to-hypha transition in *C. albicans*. Its hypha-inhibiting properties were attributed to several fatty acids, including conjugated linoleic acid (CLA) (Clement *et al.*, 2007). The following study addresses the mechanism by which CLA inhibits hyphal growth in *C. albicans*. To gain insight into the mode of action of CLA, gene expression analyses were performed. These analyses also allowed the transcriptional program regulating the yeast-to-hypha transition in Spider medium to be characterized. These findings, i.e. the hyphal growth program in Spider medium and the mechanism by which CLA blocks the yeast-to-hypha transition, are presented in this chapter, whose content has been published in an article entitled "Conjugated linoleic acid inhibits hyphal growth in *Candida albicans* by modulating Ras1p cellular levels and downregulating *TEC1* expression" in the scientific journal Eukaryotic Cell in 2011 (Shareck, J., Nantel, A. & Belhumeur, P.). Supplementary data can be found at the following address: http://ec.asm.org/cgi/content/full/10/4/565/DC1. Supplementary Tables S1 and S4 are also presented in the Appendix section.

I designed and performed most of the experiments. I grew the cells and isolated their RNA for the gene expression profiling experiments, which were designed by Nantel. Nantel also labeled the RNA, performed the hybridizations, and conducted the statistical analyses. The microscopy experiment performed to localize GFP-Ras1p was suggested by Belhumeur. I analyzed the data, assembled the figures, and wrote the manuscript, which Belhumeur and Nantel corrected. I estimate my contribution to being close to 90%.

It is notable to mention that at the time this work was undertaken, most signaling pathways involved in the regulation of the yeast-to-hypha transition had already been identified. Comprehensive reviews describing the wiring of morphogenetic signaling pathways into a complex network were published in 2007 (Biswas *et al.*, 2007; Brown *et al.*, 2007). Additionally, several molecules had been shown to modulate morphogenesis, but their mechanisms of action had seldom been investigated. The quorum sensing molecule farnesol was the best documented inhibitor of hyphal growth: its hypha-inhibiting effects had been characterized (Hornby *et al.*, 2001; Mosel *et al.*, 2005) and reported to involve a two-component signal transduction kinase (Kruppa *et al.*, 2004). Additionally,

several gene expression analyses had been performed to investigate its transcriptional effects (Cao *et al.*, 2005; Enjalbert & Whiteway, 2005). The first studies addressing farnesol's molecular mechanism of action were published in 2008 (Davis-Hanna *et al.*, 2008; Kebaara *et al.*, 2008). A receptor or a binding protein for farnesol has yet to be identified.

Conjugated linoleic acid inhibits hyphal growth in *Candida albicans* by modulating Ras1p cellular levels and downregulating *TEC1* expression

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Running title: Conjugated linoleic acid inhibits hyphal growth in *Candida albicans*

Keywords: *Candida albicans*, conjugated linoleic acid, fatty acids, yeast-to-hypha transition, hyphal growth, Ras1p signaling, Ras1p, *TUP1*, *NRG1*, *RAS1*, *TEC1, UME6*

2.1 Abstract

The polymorphic yeast *Candida albicans* exists in yeast and filamentous forms. Given that the morphogenetic switch coincides with the expression of many virulence factors, the yeast-to-hypha transition constitutes an attractive target for the development of new antifungal agents. Since an untapped therapeutic potential resides in small molecules that hinder *C. albicans* filamentation, we characterized the inhibitory effect of conjugated linoleic acid (CLA) on hyphal growth and addressed its mechanism of action. CLA inhibited hyphal growth in a dose-dependent fashion, in both liquid and solid hypha-inducing media. The fatty acid blocked germ tube formation without affecting cellular growth rates. Global transcriptional profiling revealed that CLA downregulated the expression of hypha-specific genes and abrogated the induction of several regulators of hyphal growth, including *TEC1*, *UME6*, *RFG1*, and *RAS1*. However, neither *UME6* nor *RFG1* was necessary for CLA-mediated hyphal growth inhibition. Expression analysis showed that the downregulation of *TEC1* expression levels by CLA depended on *RAS1*. In addition, while *RAS1* transcript levels remained constant in CLA-treated cells, its protein levels declined with time. With the use of a strain expressing GFP-Ras1p, CLA treatment was also shown to affect Ras1p localization to the plasma membrane. These findings suggest that CLA inhibits hyphal growth by affecting the cellular localization of Ras1p and blocking the increase in *RAS1* mRNA and protein levels. Combined, these effects should prevent the induction of the Ras1p signaling pathway. This study provides the biological and molecular explanations that underlie CLA's ability to inhibit hyphal growth in *C. albicans*.

2.2 Introduction

Over the past decades, opportunistic fungal infections have gained importance among hospital-acquired infections due to a growing community of individuals immunocompromised by HIV infection, cancer treatment, or organ transplantation (Richardson & Lass-Florl, 2008). The opportunistic pathogen *Candida albicans*, a member

of the normal human microbiota, inhabits the gastrointestinal and genitourinary tracts, mucous membranes, and skin. It is responsible for various forms of diseases, ranging from superficial infections of mucosal surfaces to severe, life-threatening systemic infections that largely depend on a host's physical and physiological conditions. *C. albicans* is the fourth leading cause of nosocomial infections and the most common fungal species causing bloodstream infections, with associated mortality rates of 38-49% (Edmond *et al.*, 1999, Wisplinghoff *et al*., 2004, Pfaller & Diekema, 2007). Treatment of such infections is complicated by a limited number of antifungal drugs, many of which have adverse side-effects, and by emerging resistance to all clinically useful antifungals.

The success of *C. albicans* as a pathogen stems from its ability to deploy a full armada of activities that contribute to its virulence, including the production of proteases, adhesins, and phospholipases as well as its ability to switch morphologically and phenotypically (Kumamoto & Vinces, 2005, Brown *et al*., 2007a). The most studied morphological switch, the yeast-to-hypha transition, is induced by a variety of environmental cues, including elevated temperature, neutral or alkaline pH, nitrogen and/or carbon starvation, and growth in serum (Ernst, 2000). A complex network of signaling pathways regulate hyphal growth (Biswas *et al*., 2007, Whiteway & Bachewich, 2007, Brown *et al*., 2007b), among which the Ras1p-cyclic AMP (cAMP)-protein kinase A (PKA) and the mitogen-activated protein (MAP) kinase pathways play major roles. Both cascades are controlled by the small GTPase Ras1p (Leberer *et al*., 2001). Membrane-bound, Ras1p binds the Ras-association domain of the adenylate cyclase Cyr1p, thereby stimulating the cyclization of ATP into cAMP (Fang & Wang, 2006). Upon binding cAMP, the regulatory subunit of PKA Bcy1p releases the catalytic subunits Tpk1p and Tpk2p, thereby activating PKA (Cassola *et al*., 2004). The transcription factor Efg1p is a downstream target of PKA (Stoldt *et al*., 1997, Bockmuhl & Ernst, 2001). Ras1p also impinges on the MAP kinase pathway, downstream of which lies the transcription factor Cph1p (Liu *et al*., 1994). Other transcription factors involved in hyphal growth include Tec1p, Flo8p, Cph2p, Ume6p, Rim101p and Czf1p (Schweizer *et al*., 2000, Lane *et al*.,

2001a, Cao *et al*., 2006, Lane *et al*., 2001b, Banerjee *et al*., 2008, Zeidler *et al*., 2009, Davis *et al*., 2000, Brown *et al*., 1999). Hyphal growth is negatively controlled by the general repressor Tup1p in association with the DNA-binding proteins Nrg1p and Rfg1p (Braun & Johnson, 1997, Murad *et al*., 2001, Braun *et al*., 2001, Kadosh & Johnson, 2001). Activation of these signal transduction cascades modulates the expression of hypha-specific genes, many of which are involved in virulence (Biswas *et al*., 2007, Brown *et al*., 2007b).

Although it has recently been demonstrated that the yeast-to-hypha transition is not always required for infectivity in systemic candidiasis (Noble *et al*., 2010), it is generally accepted that hyphal growth is critical for virulence in various types of *C. albicans* infections. Several lines of evidence link the yeast-to-hypha transition to pathogenicity, the first being that mutants locked in either yeast (*cph1/cph1*, *efg1/efg1*, and *hgc1/hgc1*) or filamentous forms (*tup1/tup1* and *nrg1/nrg1*) are avirulent in systemic candidiasis (Lo *et al*., 1997, Zheng & Wang, 2004, Braun *et al*., 2000, Murad *et al*., 2001). Filamentation is required for *C. albicans* to evade phagocytes and escape from blood vessels (Lorenz *et al*., 2004, Phan *et al*., 2000). Colonization of medical devices by biofilms depends on hyphal development and the expression of hypha-specific gene products (Nobile *et al*., 2006). In parallel, using *C. albicans* strains in which hyphal growth can be manipulated externally, several groups have shown that inhibiting filamentation is a means by which virulence may be attenuated during systemic candidiasis (Saville *et al*., 2006, Carlisle *et al*., 2009). Moreover, small molecules that block filamentation have been shown to exert a protective effect in mucosal candidiasis (Hisajima *et al*., 2008) and to reduce *C. albicans*-induced damage to endothelial cells (Toenjes *et al*., 2005). Not only do these findings demonstrate an association between filamentation and virulence in *C. albicans*, but they also suggest that the yeast-to-hypha transition may constitute a therapeutic target.

We recently isolated fatty acids from bovine whey that had the ability to inhibit germ tube formation in *C. albicans* (Clement *et al*., 2007). Given that the inability to switch to a hyphal form reduces the virulence potential of *C. albicans*, we characterized the

inhibitory effect of conjugated linoleic acid (CLA) on hyphal growth and investigated its mechanism of action. We demonstrate that CLA inhibits hyphal development in a dose-dependent fashion, in both liquid- and solid-inducing media. Global gene expression analysis reveals that CLA affects the expression of hypha-specific genes and of several morphogenesis regulators, including *RAS1*, *TEC1*, and *UME6*. We show that Ras1p is required for CLA to downregulate *TEC1* expression levels. We demonstrate that in presence of CLA, Ras1p cellular levels decrease. In addition, CLA causes the delocalization of Ras1p from the plasma membrane. These findings suggest that CLA inhibits hyphal growth in *C. albicans* by affecting Ras1p signaling and downregulating the expression of downstream targets, including *TEC1* and *UME6*.

2.3 Material and methods

2.3.1 Strains and growth conditions

The yeast strains used in this study are listed in Table II. Strains were streaked out onto YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% Bacto agar) and grown at 30°C for 24-48 h. In all of the experiments performed, strains were propagated overnight in YPD at 30°C to an optical density at 600 nm (OD_{600}) of ~12-14. Hyphal inductions were performed at 37°C on solid and in liquid media, using Spider (Liu *et al*., 1994), Lee's (Lee *et al*., 1975), and buffered alkaline (pH 8.0) M199 (Wisent). Media were solidified with 2% Bacto agar (Oxoid). When necessary, media were supplemented with uridine (50 μg ml^{-1}). Conjugated linoleic acid (CLA) (Cayman Chemicals) was diluted in ethanol as a 1,000× stock and added to media in concentrations ranging from 0 to 250 μM. Ethanol was used as drug vehicle (final concentration ≤ 0.5%).

Table II *Candida albicans* strains used in this study

Strain	Parent	Genotype	Reference
SC5314		*Candida albicans* wild type clinical isolate	Gillum *et al*. (1984)
CAI4		*ura3::λimm434/ura3::λimm434*	Fonzi & Irwin (1993)
ZK3379	CAI4	*HWP1-lacZ-URA3*	Hogan *et al*. (2004)
MRC6	CAI4	*fox2::hisG/fox2::hisG RPS10/rps10::URA3*	Ramirez & Lorenz (2007)
MRC10	CAI4	*icl1::hisG/icl1::hisG RPS10/rps10::URA3*	Ramirez & Lorenz (2007)
HLC52	CAI4	*efg1::hisG/efg1::hisG-URA3-hisG*	Lo *et al*. (1997)
CDH107	CAI4	*ras1::hisG/ras1::hisG-URA3-hisG*	Leberer *et al*. (2001)
BCa2-10	CAI4	*tup1::hisG/tup1::hisG-URA3-hisG*	Braun & Johnson (1997)
BCa23-3	CAI4	*nrg1::hisG/nrg1::hisG-URA3-hisG*	Braun *et al*. (2001)
DK129	CAI4	*rfg1::hisG/rfg1::hisG-URA3-hisG*	Kadosh & Johnson (2001)
IIHB6	CAI4	*tpk1::hisG/tpk1::hisG-URA3-hisG*	Bockmuhl *et al*. (2001)
TPO7.4	CAI4	*tpk2::hisG/tpk2::hisG-URA3-hisG*	Sonneborn *et al*. (2000)
WY-ZXD3	CAI4	*RAS1/GFP-RAS1-hisG-URA3-hisG*	Zhu *et al*. (2009)
RM1000		*ura3::λimm434/ura3::λimm434 his1::hisG/his1::hisG*	Negrego *et al*. (1997)
MRC41	RM1000	*ctf1::HIS1/ctf1::hisG RPS10/rps10::URA3*	Ramirez & Lorenz (2009)
BWP17		*ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG*	Wilson *et al*. (2000)
CJN308	BWP17	*tec1::Tn7-UAU1/tec1::Tn7-URA3*	Nobile & Mitchell (2005)
SN95		*ura3::λimm434/URA3 iro1::λimm434/IRO1 arg4/arg4 his1/his1*	Noble & Johnson (2005)
SN152	SN95	*ura3::λimm434/URA3 iro1::λimm434/IRO1 arg4/arg4 leu2/leu2 his1/his1*	Noble & Johnson (2005)
DK318	SN95	*arg4::ARG4/arg4 his1::HIS1/his1*	Banerjee *et al*. (2008)
DK312	SN152	*arg4::ARG4/arg4 leu2/leu2 his1/his1 ume6:: C.m.LEU2/ume6::C.d.HIS1*	Banerjee *et al*. (2008)

2.3.2 Hyphal growth assays in liquid and on solid media

To quantify the inhibitory effect of CLA on *C. albicans* hyphal growth, β-galactosidase activity was measured using the *C. albicans* reporter strain ZK3379 in which lacZ is under the control of the hypha-specific *HWP1* promoter (Hogan *et al*., 2004). Briefly, cells from an overnight culture were washed in sterile distilled water and diluted to 1×10^6 cells ml^{-1} in prewarmed media. Twenty-four-well polystyrene microplates (Costar 3526; Corning, NY) were seeded with 1 ml of inoculated medium per well. Working solutions of CLA were freshly prepared and added immediately to seeded wells in volumes of 5 μl, yielding final concentrations ranging from 0 to 50 μM. Microplates were incubated statically in a water bath set to 37°C for 4 h. β-galactosidase activity was quantified as described previously (Kippert, 1995). Data are represented as the means and standard deviations of results from replicate assays performed on three independent days. To assess the effect of CLA on colony morphology, *C. albicans* cells from an overnight culture were washed in sterile distilled water and spread (ca. 100 colonies per plate) on plates of solidified medium supplemented with ethanol or CLA (100 μM). Plates were incubated at 37°C for 3-4 days and photographed using a LEICA MZ FLIII Fluorescence Stereomicroscope mounted with a Micropublisher camera. To examine the effect of CLA on hyphal growth of *C. albicans* wild-type and mutant strains, cells from an overnight culture were washed in sterile distilled water and diluted to a concentration of 1×10^6 cells ml^{-1} in prewarmed Spider medium supplemented with ethanol or CLA (25 μM). Flasks were shaken at 150 RPM at 37°C. Aliquots of cells were harvested at various time points, fixed with 3.7% formaldehyde for 30 min at room temperature, washed twice with 1× phosphate-buffered saline (PBS), and visualized by differential interference contrast (DIC) microscopy using an upright Nikon microscope with a 100× immersion oil objective and a 10× projection lens mounted with a Nikon DXM1200F digital camera. To distinguish hyphae from pseudohyphae, fixed cells were washed twice in water, stained for 5 min with 4'-6'diamidino-2-phenyl-indole (DAPI; 1 mg ml^{-1}) diluted 1:1,000 and calcofluor white (1 mg ml^{-1}) diluted 1:10, examined microscopically using epifluorescence, and classified according to the criteria of Sudbery *et al*. (2004). To ensure that CLA treatment did not

compromise cellular growth rates, growth curves were generated. SC5314 cells from an overnight culture were diluted to 1×10^6 cells ml^{-1} in Spider medium supplemented with ethanol or CLA (25 μM). Flasks were shaken at 150 RPM at 30°C. OD_{600} was measured every hour for 7 h. Data are means and standard deviations of results from duplicate biological samples. The effect of CLA (250 μM) on cellular growth rates in YPD at 30°C was assessed similarly.

2.3.3 Gene expression profiling

SC5314 cells grown overnight in YPD at 30°C were washed in sterile distilled water and diluted to 5×10^6 cells ml^{-1} (OD_{600} of 0.1) in Spider medium supplemented with ethanol or CLA (100 μM). Cultures were shaken at 150 RPM at either 30°C or 37°C for 90 min. Cells were collected by vacuum filtration on 0.45-μm membrane filters (MF-Millipore Membrane Filters) and frozen in an ethanol bath at -80°C. Total RNA was isolated from quadruplicate independent biological samples using an RNeasy mini kit (Qiagen). Briefly, frozen cells were thawed out in RNeasy buffer RLT at a ratio of 3:1 (vol/vol) buffer/pellet. Resuspended cells were divided into 1-ml aliquots in 2-ml screw-cap microcentrifuge tubes containing 0.6 ml of 0.5-mm-diameter acid-washed glass beads. Samples were homogenized 6 times, for 5 min each, in a BeadBeater set at maximum speed. Lysates were kept on ice between each cycle. Total RNA was extracted from homogenized samples according to the Qiagen RNeasy protocol. For the two-color microarray experiments, RNA from cells grown at 37°C, or in the presence of CLA at 30°C or 37°C was compared to RNA from cells grown at 30°C. Four biological replicates were used in each experiment, which included two Cy3/Cy5 and two Cy5/Cy3 comparisons. cDNA labeling, microarray hybridization, washing, scanning, and statistical analysis methods were essentially performed as described previously (Sellam *et al*., 2009). In each comparison, genes with statistically significant modulations were identified in Volcano Plots using a 2.0-fold cut-off point and a Welsh *t* test with a false-discovery rate of less than 5%.

2.3.4 Northern blot analysis

Cells were grown as described above for gene expression profiling. RNA was prepared using the hot-phenol method. Fifteen micrograms of total RNA was separated on 1.2% agarose-7.5% formaldehyde denaturing gels and transferred by capillary action to Hybond-N^+ nylon membranes (GE Healthcare Life Sciences). Probes were generated by PCR amplification of genomic DNA and purified using an illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences). The sequences of the primers used to generate all probes are listed in Table III. Fifty nanograms of probe was labeled by random priming using Ready-To-Go DNA Labeling Beads (GE Healthcare Life Sciences) and [α-^{32}P]dCTP. Unincorporated nucleotides were removed using Sephadex G-50 columns. Blots were hybridized overnight and washed at 65°C according to the method of Church and Gilbert (Church & Gilbert, 1984), scanned using a Molecular Dynamics Typhoon phosphorimager, and quantified with ImageQuant software (version 5.0; Molecular Dynamics). Data are means and standard deviations of results from duplicate biological samples.

2.3.5 Quantitative PCR analysis of *C. albicans* transcripts

For quantitative PCR (qPCR) analysis, *C. albicans* cells from an overnight culture were diluted to 1×10^6 cells ml^{-1} in Spider medium supplemented with ethanol or CLA (25 μM) and grown at 37°C. Cells were collected at various time points (0, 30, 60, and 90 min) by vacuum filtration on 0.45-μm membrane filters (MF-Millipore Membrane Filter) and frozen in an ethanol bath at -80°C. Total RNA was isolated from duplicate independent biological samples for each condition and time point using the hot-phenol method. RNA was resuspended in 50 to 200 μl diethyl pyrocarbonate-treated water, quantified by spectrophotometer (NanoDrop 2000; Thermo Scientific), and stored at -80°C. RNA samples were DNase digested (rDNase I; Ambion) and used as templates in qPCR amplification reactions to certify them as DNA free. The lack of a PCR product indicated that samples were not contaminated with genomic DNA. Five hundred nanograms of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen)

according to the manufacturer's instructions. Two cDNAs were synthesized for each biological replicate. Quantitative PCR was carried out on a Rotor-Gene 6000 (Corbett Life Science). The primers were designed with PrimerQuest (Integrated DNA Technologies) and are listed in Table III. The qPCR mixtures contained 12.5 µl FastStart SYBR Green Master (Roche Applied Science), 8.5 µl Milli-Q water, 200 nM each primer, and 1 µl cDNA product diluted 1:100, and the qPCRs were performed in duplicates. Generally, the difference between two threshold cycle (C_T) values for the same sample was <0.5. Relative expression levels were calculated using the delta-delta C_T method [$2^{(C_T \text{ for target condition} - C_T \text{ for } \textit{ACT1} \text{ condition}) - (C_T \text{ for target at time zero} - C_T \text{ for } \textit{ACT1} \text{ at time zero})}$], in which the condition was either ethanol or CLA treatment and *ACT1* was the housekeeping gene.

Table III Primers used in this study

Primer	Sequence (5'-3')	Product size
qPCR ACT1F	5'-TCCAGA AGCTTTGTTCAGACCAGC-3'	170 bp
qPCR ACT1R	5'-TGCATACGTTCAGCAATACCTGGG-3'	
qPCR RAS1F	5' GTTGTTGTTGGAGGTGGTGGTGTT 3'	180 bp
qPCR RAS1R	5' GGCCAGATATTCTTCTTGTCCAGC 3'	
qPCR TUP1F	5'-CCAGCACCAACAACGTTTGACAGA-3'	176 bp
qPCR TUP1R	5'-TGGGCCAACTCCAAGTCATACACT-3'	
qPCR NRG1F	5'-TGGTGATTTACTGGCCAACTCCCT-3'	180 bp
qPCR NRG1R	5'-CATGTTGGCCATGGACATTGGTGT-3'	
ACT1F probe	5'-GTTGACCGAAGCTCCAATGAATCC-3'	629 bp
ACT1R probe	5'-TGCATACGTTCAGCAATACCTGGG-3'	
TEC1F probe	5'-GTTACCACCACGAGCACTGGC-3'	486 bp
TEC1R probe	5'-TGAAGGGTGTTGGCTATTATGCG-3'	

2.3.6 Protein extraction and immunoblotting

Wild-type and tagged strains were grown in Spider medium and harvested by following the same procedure as that described above for gene expression profiling. Total protein extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate [DOC], 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Complete EDTA-Free tablets [Roche Applied Science]). Total extracts (50 μg) were resolved by SDS-PAGE (7.5%) and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (GE Healthcare Life Sciences). To detect green fluorescent protein (GFP)-Ras1p, mouse anti-GFP antibodies (Roche Applied Science) (1:1,000 in Tris-buffered-saline-Tween [TBS-T]-5% nonfat milk) were used. Gsp1p protein levels, shown as a loading control, were detected using rabbit anti-Gsp1p antibodies (Belhumeur *et al*., 1993) (1:10,000 in TBS-T-5% nonfat milk) overnight at 4°C. Signals were detected using Lumi-Light Western Blotting Substrate (Roche Applied Science).

2.3.7 Microarray data accession number

Microarray data sets can be found at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE25822.

2.4 Results

2.4.1 CLA inhibits hyphal growth in *C. albicans* in response to various hypha-inducing conditions

CLA inhibits hyphal growth in *C. albicans* in response to various hypha-inducing conditions. Several fatty acids, including conjugated linoleic acid (CLA), were recently shown to inhibit *Candida albicans* germ tube formation in various hypha-inducing liquid media (Clement *et al*., 2007). We first assessed the inhibitory effect of CLA on hypha formation induced in different conditions using the *C. albicans* reporter strain *HWP1p*-lacZ

in which β-galactosidase activity reflects the amount of hyphal growth in cultures (Hogan *et al*., 2004). In Spider, Lee's, and M199 (pH 8.0) media, CLA inhibited hyphal growth in a dose-dependent fashion, albeit to various extents (Figure 2.1A). In all media, 50 μM of the fatty acid reduced β-galactosidase activity by more than 80% compared to the level for ethanol-containing cultures, confirming that the presence of CLA impeded filamentation. In Spider medium supplemented with 25 μM CLA, cells grew as yeasts and short pseudohyphae, reflecting reduced β-galactosidase activity levels (Figure 2.1A and B). CLA also disrupted filamentation on solid media, being more effective in Spider medium (Figure 2.1C). However, hyphal growth inhibition by CLA was medium-dependent. For instance, in RPMI 1640 and YPD-10% fetal bovine serum (FBS) liquid media, CLA inhibited filamentation, but effective concentrations tended to be higher (Clement *et al*., 2007; data not shown). In addition, CLA had no effect on hyphal growth induced on solid YPD-10% FBS, synthetic low ammonia dextrose (SLAD), or yeast nitrogen base (YNB) supplemented with N-acetylglucosamine or upon embedding of cells in yeast extract-peptone (YP) medium (data not shown).

Other fatty acids, such as oleic, linoleic, α- and γ-linolenic acids also modulated hyphal growth in *C. albicans*. Levels of β-galactosidase activity, as well as cellular and colony morphology, indicated that all fatty acids, like CLA, interfered to different extents with *C. albicans* hyphal growth induced under various conditions (data not shown). In addition, fatty acids, including CLA, inhibited filamentation in *Candida tropicalis* and *Candida dubliniensis* (data not shown), in *Aspergillus fumigatus* and in *Fusarium graminearum* (Clement *et al*., 2008); data not shown), suggesting the response to fatty acids and to CLA may be conserved among other fungi.

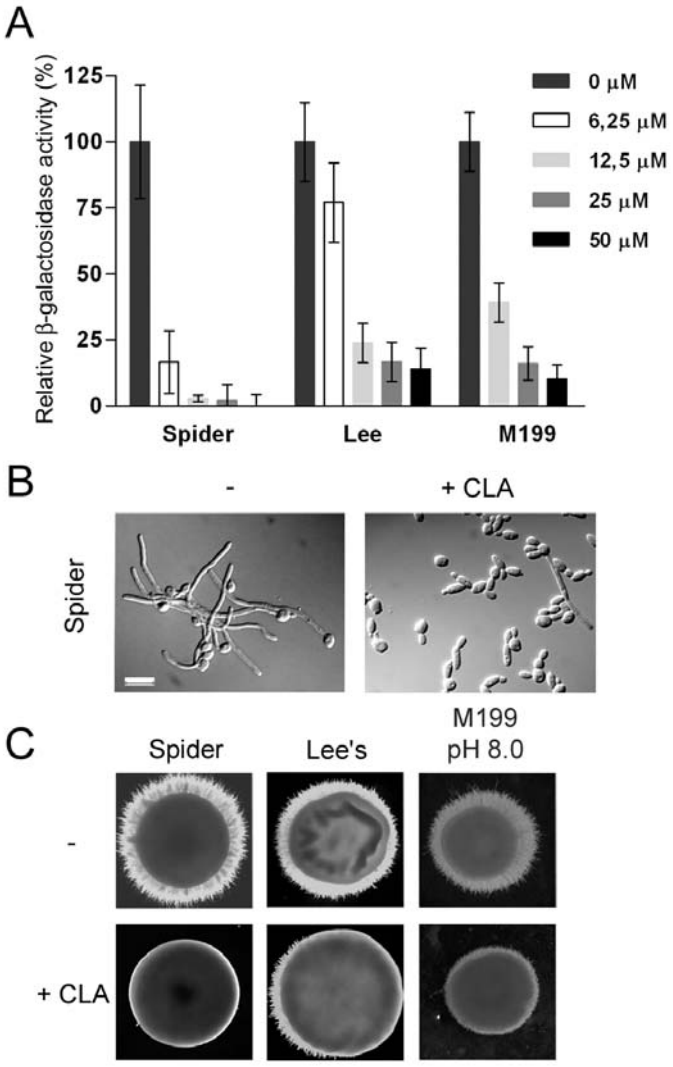

Figure 2. 1 Conjugated linoleic acid (CLA) inhibits hyphal growth in *Candida albicans*.

(A) The effect of CLA on *C. albicans* hyphal growth was measured by using the *C. albicans* reporter strain ZK3379. Cells were induced to filament in Spider, Lee's, and M199 (pH 8.0) media supplemented with CLA (0 to 50 μM) for 4 h at 37°C. β-galactosidase activity in CLA-treated cultures was measured and normalized to that of untreated cultures, in which β-galactosidase activity was set as 100%. Data are means and standard deviations of results from duplicate assays performed on three independent days. (B) Aliquots of cells grown in Spider medium in the absence or presence of 25 μM CLA for 4 h at 37°C were harvested and visualized at ×100 magnification using DIC optics. Bar = 10 μm. (C) Filamentous growth of *C. albicans* SC5314 was induced on solid media supplemented with ethanol or 100 μM CLA. Plates were incubated at 37°C for 3 to 4 days.

2.4.2 CLA impedes germ tube formation without affecting cellular growth

With the use of the *HWP1p*-lacZ strain and β-galactosidase quantification, CLA and other fatty acids were shown to abrogate, delay, or decrease the induction of *HWP1* promoter activity, indirectly suggesting that hyphal growth was impaired (Figure 2.1A) (Clement *et al*., 2007). To assess which step of the yeast-to-hypha transition was affected by CLA, we examined the morphology of cells induced to filament in Spider medium in the absence or presence of CLA. Untreated cells showed long germ tubes and/or very short filaments and long hyphae at the 1- and 3-h time points, respectively. At the same time points, most CLA-treated cells appeared as elongated yeasts or showed elongating buds that resembled germ tubes (Figure 2.2A). With the use of the criteria established by Sudbery *et al*. (2004), cells were stained with DAPI and calcofluor white and classified as yeast, pseudohyphae, or germ tubes/hyphae. As seen in Fig. 2B, 80-90% of untreated cells formed hyphae and pseudohyphae over the 3-h time course. In contrast, the majority (70 to 80%) of cells grown in the presence of CLA remained in the yeast form. While some CLA-treated cells did initiate germ tube formation, elongating filaments were not detected by the 3-h time point, indicating that these cells had resumed pseudohyphal and yeast growth modes. These findings suggest that CLA prevents germ tube formation and hyphal elongation from occurring. CLA also blocked hyphal elongation in cells that were already engaged in the hyphal growth program (data not shown).

C. albicans filamentation can be inhibited by cytotoxic or cytostatic molecules (Toenjes *et al*., 2005). Since CLA interfered with hyphal growth, we assessed whether cellular growth rates at 30°C in Spider medium were affected by ethanol or CLA. In these conditions, germ tube formation is slightly induced at early time points (Figure 2.3A), but hyphal growth is not maintained and cells eventually resume budding growth (data not shown). The growth rates of untreated and CLA-treated cells were identical, suggesting that CLA does not inhibit hyphal growth by exerting cytotoxic or cytostatic effects (Figure

2.2C). Similar results were obtained for yeast cells grown in the absence or presence of CLA in YPD medium (data not shown).

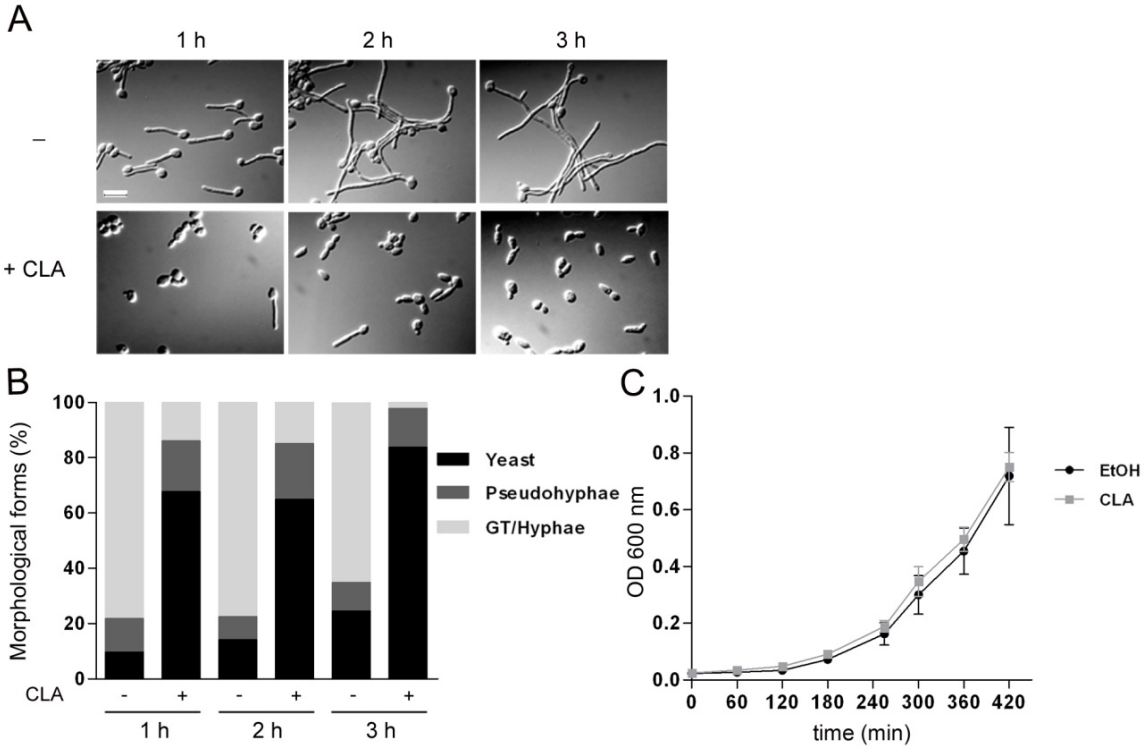

Figure 2. 2 CLA impedes germ tube formation of *Candida albicans* without affecting cellular growth.

(A) *C. albicans* SC5314 cells were induced to filament in Spider medium at 37°C in the absence or presence of 25 µM CLA. Aliquots of cells were visualized as described for Figure 2.1B. Bar = 10 µm. (B) Quantification of yeast, pseudohyphae, germ tubes (GT) and hyphae in *C. albicans* cultures shown in panel A. *n* was >150 for each condition and time point. (C) Yeast cells were grown as described for panel A at 30°C. OD_{600} was measured at various time points. Data are means and standard deviations of results from duplicate biological samples.

2.4.3 Gene expression analysis

To gain further insight into the inhibitory effect of CLA on hyphal growth, we performed global gene expression profiling of cells grown in Spider medium in the absence or presence of CLA for 90 min. Transcriptional profiles of untreated cells at 37°C and CLA-treated cells at 37°C and 30°C were obtained by independently comparing the levels for each experimental condition, i.e., 37°C, 37°C with CLA, and 30°C with CLA, to those for the control condition, growth at 30°C. Each experiment was performed in quadruplicate: RNA was prepared from four independent biological replicates and used to perform four independent hybridizations (Figure 2.3A). Only genes that were modulated 2-fold up or down with a *P* value <0.05 were deemed significantly differentially expressed. Data presented in Tables S1, S2, and S3 in the supplemental material show the transcriptional profiles of each experimental condition compared to the control condition. Transcriptional profiles of the experimental conditions were then compared to one another, i.e., 37°C versus 37°C with CLA and 30°C with CLA versus 37°C with CLA, resulting in two other transcriptional profiles (Table S4 and S5) and the scatter plots in Figure S1A. All significantly differentially expressed genes originating from the five transcriptional profiles generated were organized by hierarchical clustering, yielding a global transcriptional profile of 714 modulated transcripts (Table S6 and Figure S1B). The hierarchical clustering revealed that 61 genes were downregulated under all three experimental conditions (Figure S1B). Gene ontology (GO)-term analysis revealed these genes were involved in RNA metabolic processes, ribosome biogenesis, translation, and transcription, reflecting a repression of the translational machinery. These findings suggest that experimental growth conditions, i.e., 37°C and CLA treatment, are less favorable than control growth conditions, i.e., 30°C, and result in a metabolic decrease (Nantel *et al*., 2002).

We first chose to investigate the transcriptional profile of cells grown in Spider medium at 37°C. Gene expression analysis showed that 520 genes were modulated upon growth at 37°C (see Table S1 in the supplemental material). Of those genes, 198 were upregulated while 322 were downregulated. Interestingly, ~30% of the upregulated genes

have been described as being induced during the yeast-to-hypha transition in Lee's medium (Goyard *et al*., 2008) (Table S1). Hypha-specific genes such as *ECE1*, *RBT1*, *IHD1*, *SOD5*, *HWP1*, *ALS3*, and *HGC1* were highly induced (Nantel *et al*., 2002, Goyard *et al*., 2008, Zheng & Wang, 2004). Genes involved in signal transduction including the GTPase *RAS1*, the adenylate cyclase *CYR1*, the mucin-like signaling protein gene *MSB2*, and the Rho1p GTPase-activating protein (GAP) gene *BEM2*, were also upregulated (Tables IV and S1). In addition, several transcription factors known to be involved in hyphal growth were among the 198 upregulated genes and included *CPH1*, *CPH2*, *TEC1*, *BCR1*, and *UME6* (Table IV). These transcription factors are components of the MAP kinase, the Ras1p-cAMP-PKA, and the Tup1p-Nrg1p signaling pathways (Liu *et al*., 1994, Lane *et al*., 2001b, Lane *et al*., 2001a, Nobile & Mitchell, 2005, Kadosh & Johnson, 2005, Banerjee *et al*., 2008, Zeidler *et al*., 2009), except *CPH2*, which appears to function independently of known signaling cascades (Lane *et al*., 2001b). Other upregulated genes encoded transcription factors Gat2p and Cas4p (Goyard *et al*., 2008) while orf19.6705 encodes a nucleotide exchange factor. Taken together, the transcriptional profile of cells grown at 37°C suggests that several signaling pathways participate in orchestrating the hyphal growth program in Spider medium.

Next, we examined the responses of cells to CLA at 30°C and 37°C. We had designed the microarray experiments to be able to distinguish between the transcriptional changes associated with morphological differences induced by growth at 37°C and those elicited specifically by the fatty acid. However, cultures of *C. albicans* grown in Spider at 30°C still contained a significant number of pseudohyphal cells (Figure 2.3A), making it impossible to fully eliminate morphogenesis as a variable. Thus, transcriptional profiles of CLA's effects at both temperatures turned out to be highly similar, as seen in the scatter plot (see Figure S1A in the supplemental material). Indeed, when the transcriptional profiles of CLA-treated cells at 37°C and those at 30°C were directly compared, only 33 genes were differentially modulated (see Table S5 in the supplemental material), and many of these have previously been shown to be temperature regulated (Enjalbert *et al*., 2003).

Table IV Selected genes upregulated during the yeast-to-hypha transition in Spider medium

Systematic name	Gene name	Function	Fold-change
orf19.1187	*CPH2*	Transcriptional activator of hyphal growth	3.0
orf19.6705		Putative guanyl nucleotide exchange factor with Sec7p domain	2.9
orf19.4056	*GAT2*	Putative DNA-binding transcription factor	2.8
orf19.5908	*TEC1*	TEA/ATTS transcription factor involved in regulation of hypha-specific genes	2.7
orf19.1822	*UME6*	Transcription factor; required for wild-type hyphal extension	2.7
orf19.1693	*CAS4*	Protein of RAM cell wall integrity signaling network; role in cell separation; required for hyphal growth	2.5
orf19.723	*BCR1*	Transcription factor required for wild-type biofilm formation	2.3
orf19.4433	*CPH1*	Transcription factor required for mating and hyphal growth on solid media	2.1
orf19.5148	*CYR1*	Adenylate cyclase	2.0
orf19.1760	*RAS1*	RAS signal transduction GTPase; regulates cAMP and MAP kinase pathways	2.1
orf19.1490	*MSB2*	Mucin family member, possible sensor of cell wall damage	3.4
orf19.6573	*BEM2*	Putative Rho1p GTPase-activating protein (GAP)	2.4

We thus focused our functional analysis on the transcriptional profile of cells exposed to CLA at 37°C compared to cells grown at 30°C. CLA treatment resulted in the modulation of 296 transcripts (Table S2). One hundred fifty-five genes were upregulated while 141 were downregulated. GO-term analysis showed that upregulated genes belonged to the categories “unknown biological function” (31%), “response to chemical stimulus” (17.4%), “carbohydrate metabolic processes” (16.8%), and “lipid metabolic processes” (16.1%). Fatty acids are nonfermentable carbon sources which are converted to acetyl-

coenzyme A (acetyl-CoA) by β-oxidation. Acetyl-CoA drives the glyoxylate cycle, yielding oxaloacetate, which is converted to glucose via gluconeogenesis. Transcript levels

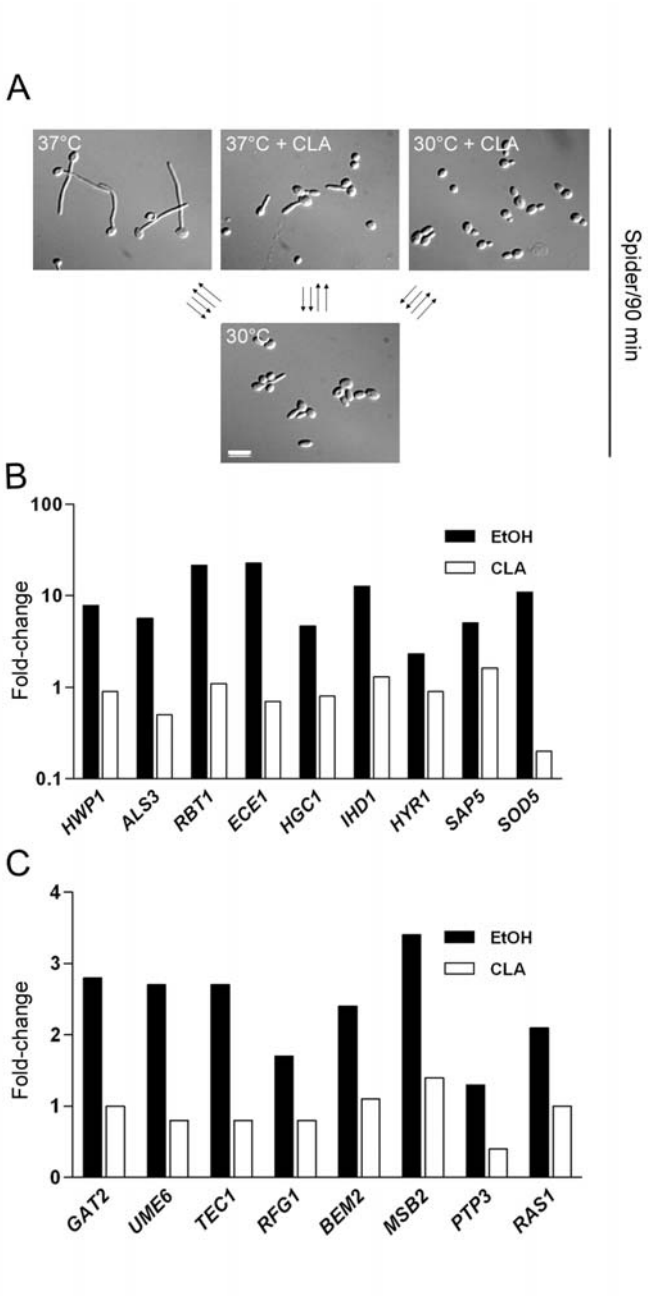

Figure 2. 3 Expression levels of selected differentially expressed genes.

(A) Transcriptional profiles of untreated cells at 37°C and CLA-treated cells at 37°C and 30°C were obtained by independently comparing the levels for each experimental condition, i.e., 37°C, 37°C with CLA and 30°C with CLA, to those for the control condition, growth at 30°C. Four biological replicates were used in each experiment, which included two Cy3/Cy5 and two Cy5/Cy3 comparisons. Representative micrographs of cells used in the microarray experiments. Bar = 10 μm. Fold change values are shown for hypha-specific genes (B) and genes involved in signal transduction (C) in untreated and CLA-treated cells at 37°C. The significantly differentially expressed genes were obtained by comparing the transcriptional profile of cells at 37°C with that of CLA-treated cells at 37°C and are listed in Table S4 in the supplemental material. EtOH, ethanol.

of hallmark genes of the β-oxidation (*FAA21*, *POX1*, *PXP2*, *POX1-3*, *ECI1*, *POT1*, *FOX2*, *FOX3*, *PEX5*, *CAT2*, and *ANT1*), glyoxylate (*ICL1*, *MLS1*, and *MDH1-3*), and gluconeogenesis (*FBP1*) pathways increased in the presence of CLA, which may reflect a flow of carbon from fatty acids to acetyl-CoA to glucose. Most of these genes have been described as being induced upon internalization of *C. albicans* by macrophages (Lorenz *et al*., 2004) or by oleic acid (Ramirez & Lorenz, 2009). Genes encoding glycolytic enzymes, including *PGK1*, *PGI1*, and *FBA1*, were also upregulated in the presence of CLA, which may suggest that both glycolysis and gluconeogenesis are occurring simultaneously. In addition, a group of genes involved in transport (15.5%), including *TPO4*, *PDR16*, *CDR11*, *CDR4*, *RTA3*, and *FLU1*, were upregulated in the presence of CLA. These genes encode transmembrane transporters which play roles in phospholipid, fatty acid, or drug transmembrane transport.

To address the mechanism of action of CLA, we compared the transcriptional profiles of cells grown in the absence or presence of CLA at 37°C. Gene expression analysis revealed that 150 genes were significantly differentially expressed (see Table S4 in the supplemental material). To facilitate data mining, fold change ratios were generated by normalizing data obtained for CLA-treated cells to that obtained for untreated cells, resulting in 72 and 78 genes with upregulated (>2-fold) and downregulated (<0.5-fold) fold change ratios, respectively (Table S4). GO-term analysis revealed the subset of 72 CLA-

upregulated genes was enriched in genes implicated in "lipid metabolic processes" (27.4%) and "transport" (15.1%). Most of the genes involved in transmembrane transport, fatty acid β-oxidation and peroxisome biogenesis, the glyoxylate cycle, and gluconeogenesis were among the genes induced in CLA-treated cells (Table S2) discussed in the previous section. Because cells may be metabolizing CLA and converting it to glucose, we examined whether the CLA-mediated inhibition of hyphal growth was linked to fatty acid metabolism. To do so, we analyzed the effect of CLA on filamentation of the *fox2/fox2*, *icl1/icl1*, and *ctf1/ctf1* mutant strains, which filament normally, but cannot assimilate fatty acids (Ramirez & Lorenz, 2007, Ramirez & Lorenz, 2009). CLA inhibited hyphal growth in all three strains, suggesting that fatty acid metabolism is not involved in the inhibition of filamentation (data not shown).

Given that lipid metabolism was not impeding hyphal growth, we focused on the subset of 78 CLA-downregulated genes (see Table S4 in the supplemental material). GO-term analysis showed that genes involved in "filamentous growth" (20.8%) and "pathogenesis" (16.9%) were among the subset of CLA-downregulated genes. Indeed, expression levels of hypha-specific genes, including *HWP1*, *ALS3*, *RBT1*, *ECE1*, *HGC1*, *IHD1*, *HYR1*, *SAP5*, and *SOD5*, were greatly reduced in CLA-treated cells compared to the levels for untreated cells (Figure 2.3B and Table S4). CLA also blocked the induction of transcription factors involved in hyphal growth, such as *GAT2*, *UME6*, *TEC1*, and *RFG1* (Figure 2.3C). A smaller (5.2%) yet more interesting category of CLA-downregulated transcripts was enriched in genes involved in signal transduction, such as *RAS1*, *BEM2*, *MSB2*, and *PTP3* (Figure 2.3C). As seen in Table III, transcript levels of these genes increased during the yeast-to-hypha transition, except for *PTP3* (Table S4). CLA either prevented the induction or lowered the induction levels of *RAS1*, *BEM2*, and *MSB2*. As for *PTP3*, which encodes a protein tyrosine phosphatase, its transcript levels were repressed in CLA-treated cells (Figure 2.3C and Table S4). Taken together, the transcriptional data suggest that CLA negatively affects the expression of hypha-specific genes as well as genes known to regulate the yeast-to-hypha transition.

2.4.4 *UME6* and *RFG1* are not required for CLA-mediated inhibition of hyphal growth

Gene expression analysis revealed that CLA reduced the expression levels of genes encoding regulators of hyphal growth, including *TEC1*, *UME6*, *RFG1*, and *RAS1* (Figure 2.3C). To determine whether these regulators were required for CLA's effect on hypha formation, we examined the responses of *ume6/ume6* and *rfg1/rfg1* mutant strains to CLA when induced to filament in Spider medium at 37°C. As seen in Figure 2.4, CLA inhibited hyphal growth in all parental strains. Interestingly, CLA inhibited filamentation independently of *UME6* and *RFG1*, indicating that neither gene is required for CLA's inhibitory effect on hyphal growth (Figure 2.4). The *ras1/ras1* and *tec1/tec1* mutants could not be analyzed in a similar fashion, since these strains failed to filament in Spider medium as previously reported (our observations; Leberer *et al*., 2001, Homann *et al*., 2009, Zhu *et al*., 2009).

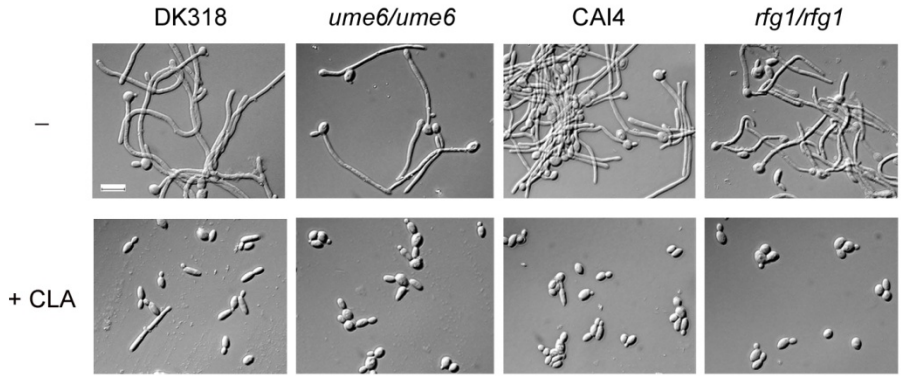

Figure 2. 4 *UME6* and *RFG1* are not required for CLA-mediated hyphal growth inhibition.

The DK318, CAI4, *ume6/ume6* (DK312), and *rfg1/rfg1* (DK129) strains were grown in Spider medium at 37°C in the absence or presence of 25 μM CLA for 4 h. Cells were visualized as described for Figure 2.1B. Bar = 10 μm.

2.4.5 CLA downregulates *TEC1* expression in a Ras1p-dependent manner

Based on the previous screen, morphogenesis regulators potentially mediating CLA's effect on hyphal growth were narrowed down to Ras1p and Tec1p, since both were required for filamentation in Spider medium (data not shown). In its active GTP-bound form, Ras1p activates the adenylate cyclase Cyr1p, stimulating the cyclization of ATP to cAMP (Fang & Wang, 2006). Upon binding cAMP, Bcy1p, the regulatory subunit of PKA, releases the functionally redundant catalytic subunits Tpk1p and Tpk2p, thereby enabling their activity (Cassola *et al*., 2004). The transcription factor Efg1p, an important regulator of hyphal growth, is a downstream target of PKA (Bockmuhl & Ernst, 2001). In addition, Efg1p also regulates *TEC1* expression (Lane *et al*., 2001a). Thus, Tec1p constitutes a downstream target of the Ras1p-cAMP-PKA pathway.

Gene expression analysis showed that CLA reduced *TEC1* expression levels (Figure 2.3C). Given that *TEC1* is induced upon the yeast-to-hypha transition, we examined how CLA affected the kinetics of expression of *TEC1*. We performed a time course analysis of TEC1 transcript levels in cells grown in Spider medium in the absence or presence of CLA. As expected, *TEC1* expression was induced in untreated cells during the yeast-to-hypha transition (Figure 2.5A). In contrast, *TEC1* mRNA levels were downregulated by an average of 5-fold in CLA-treated cells compared to the level for untreated cells, thus confirming transcriptional profiling results. Similar trends were also observed with the use of quantitative PCR analysis (data not shown). Furthermore, Tec1p protein levels were reduced in the presence of CLA, thus following the same expression pattern as the *TEC1* transcript (data not shown).

A possible role for the Ras1p-cAMP-PKA signaling pathway in mediating CLA's effect on *TEC1* expression was then investigated. Northern blot analysis revealed that in Spider medium, *TEC1* induction was mostly Ras1p-dependent (Figure 2.5B and C).

Interestingly, the effect of CLA on *TEC1* expression was reduced in the absence of *RAS1*. In the parental strain, *TEC1* transcript levels decreased by ~10-fold in CLA-treated cells compared to the level for untreated cells, while in the *ras1/ras1* mutant strain, the downregulation only reached 1.5-fold (Figure 2.5C). On the other hand, *TEC1* induction in Spider medium and its downregulation by CLA did not depend on the presence of either *TPK1* or *TPK2* (Figure 2.5B and C). These results may be explained by the functional redundancy of the two PKA isoforms (Bockmuhl *et al*., 2001). Since Efg1p is a regulator of *TEC1* expression, we examined its role in mediating CLA's effect on *TEC1* mRNA levels. Northern analysis revealed that *TEC1* induction in Spider medium depended partially on *EFG1*, as *TEC1* transcript levels were 1.7-fold lower in the *efg1/efg1* mutant strain compared to the parental strain (Figure 2.5B and C). However, *EFG1* was not required for CLA's repressive effect on *TEC1*, as the magnitudes of *TEC1* downregulation by CLA (~2-fold) were similar in parental and mutant strains (Figure 2.5C). Additionally, it should be noted that CLA did not modulate *EFG1* mRNA and protein levels (data not shown). Taken together, these results implicate *RAS1*, but not *TPK1*, *TPK2*, or *EFG1*, in mediating CLA's repressive effect on *TEC1* expression.

2.4.6 CLA reduces GFP-Ras1p protein levels and affects its localization

Transcriptional profiling showed that *RAS1* expression was upregulated 2.1-fold during the yeast-to-hypha transition but that it was not induced in CLA-treated cells (Figure 2.3C). We examined CLA's effect on *RAS1* transcription at earlier time points by performing a time course analysis of *RAS1* mRNA levels in cells grown in Spider medium in the absence or presence of CLA. Quantitative PCR analysis revealed that *RAS1* transcript levels increased in untreated cells by an average of 2.7-fold, confirming results obtained by gene expression analysis (Figure 2.6A). On the other hand, CLA completely abrogated *RAS1* induction, as its mRNA levels remained relatively unchanged compared to levels at the zero time point (Figure 2.6A). We next investigated CLA's effect on Ras1p protein levels and localization using the WY-ZXD3 strain expressing a GFP-Ras1 fusion protein

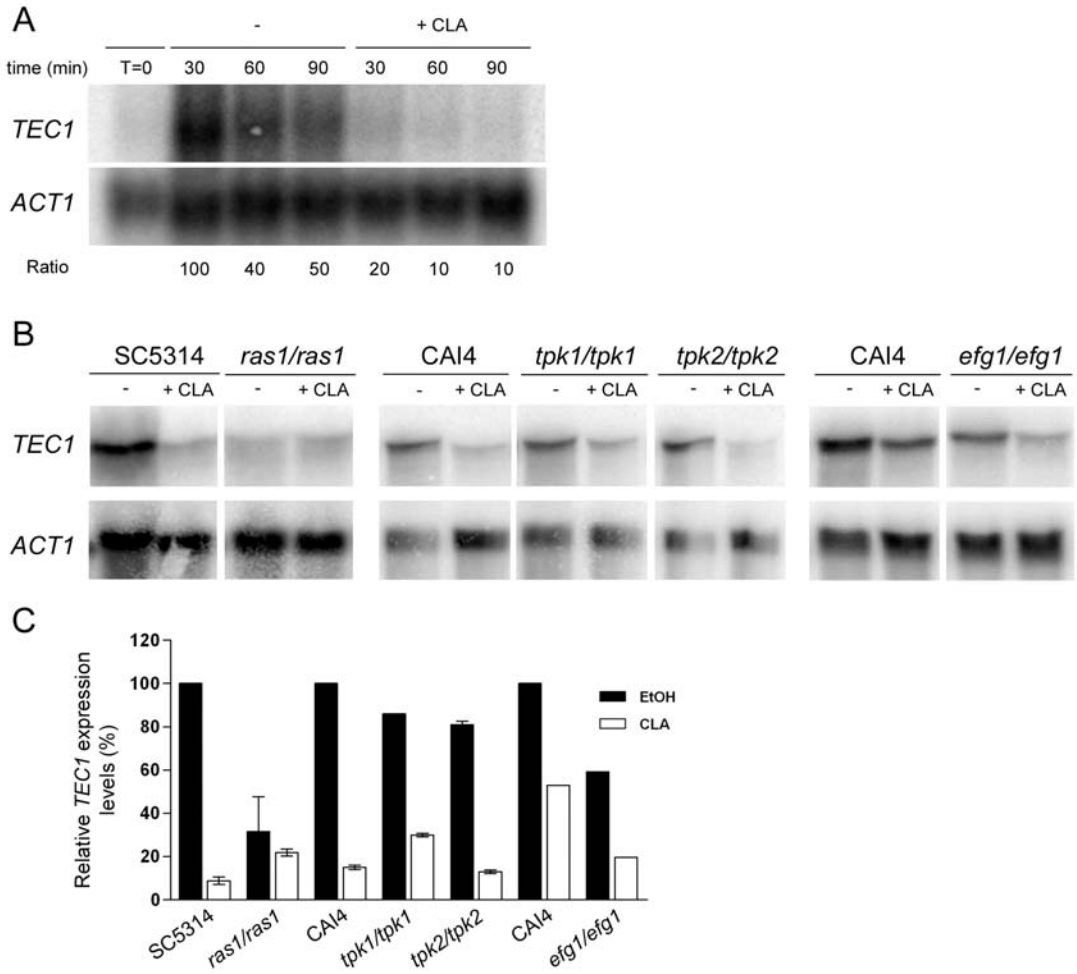

Figure 2. 5 *TEC1* downregulation by CLA is Ras1p-dependent.

(A) Quantitative Northern blot analysis was used to examine the kinetics of expression of *TEC1* in SC5314 cells grown in Spider medium at 37°C in the absence or presence of 100 μM CLA. *TEC1* transcript levels were quantified and normalized to those for the *ACT1* loading control. Ratios were obtained by normalizing *TEC1* transcript levels to those for untreated cells at the 30-min time point, which were set as 100%. (B) *TEC1* expression levels in the parental, *ras1/ras1* (CDH107), *tpk1/tpk1* (IIHB6), *tpk2/tpk2* (TPO7.4), and *efg1/efg1* (HLC52) strains grown as described for panel A for 90 min. *TEC1* transcript levels were quantified and normalized to those for the *ACT1* loading control. (C) Data presented are the relative *TEC1* expression levels obtained by normalizing *TEC1* transcript levels to those for parental untreated cells, which were set as 100%. Data are means and standard deviations of results from two independent hybridizations performed on duplicate biological samples. A single hybridization was performed using RNA isolated from the CAI4 and *efg1/efg1* strains.

previously developed for similar analyses (Zhu *et al*., 2009). We monitored GFP-Ras1p cellular levels in cells induced to filament in Spider medium in the absence or presence of CLA. In untreated cells, GFP-Ras1p levels increased gradually, as did its mRNA levels (Figure 2.6B). Unexpectedly, in CLA-treated cells, GFP-Ras1p levels declined with time, as seen at the 90-min time point (Figure 2.6B). Thus, GFP-Ras1p did not follow the same pattern as its transcript which remained constant (Figure 2.6A). Taken together, these results suggest that CLA treatment reduces the steady-state levels of GFP-Ras1p.

We then examined GFP-Ras1p localization in untreated and CLA-treated cells. To facilitate comparisons, we examined GFP-Ras1p in yeast cells grown in Spider medium in the absence or presence of CLA at 30°C. Under such conditions, GFP-Ras1p expression patterns were identical to those at 37°C, arguing that CLA's effect on GFP-Ras1p levels was not temperature dependent (data not shown). As seen in Figure 2.6C (top panel), untreated cells demonstrated a strong fluorescent signal at the plasma membrane, confirming previous observations (Zhu *et al*., 2009). Additionally, GFP-Ras1p could be seen in punctate patches within cells, especially at the 30-min time point. In contrast, GFP-Ras1p could barely be detected at the plasma membrane in CLA-treated cells. Instead, the fluorescent signal appeared diffuse throughout the cytoplasm or concentrated in patches within cells (Figure 2.6C). Moreover, the "patch" phenotype was maintained, becoming more obvious at later time points (data not shown). These findings indicate that CLA causes the delocalization of GFP-Ras1p from the plasma membrane.

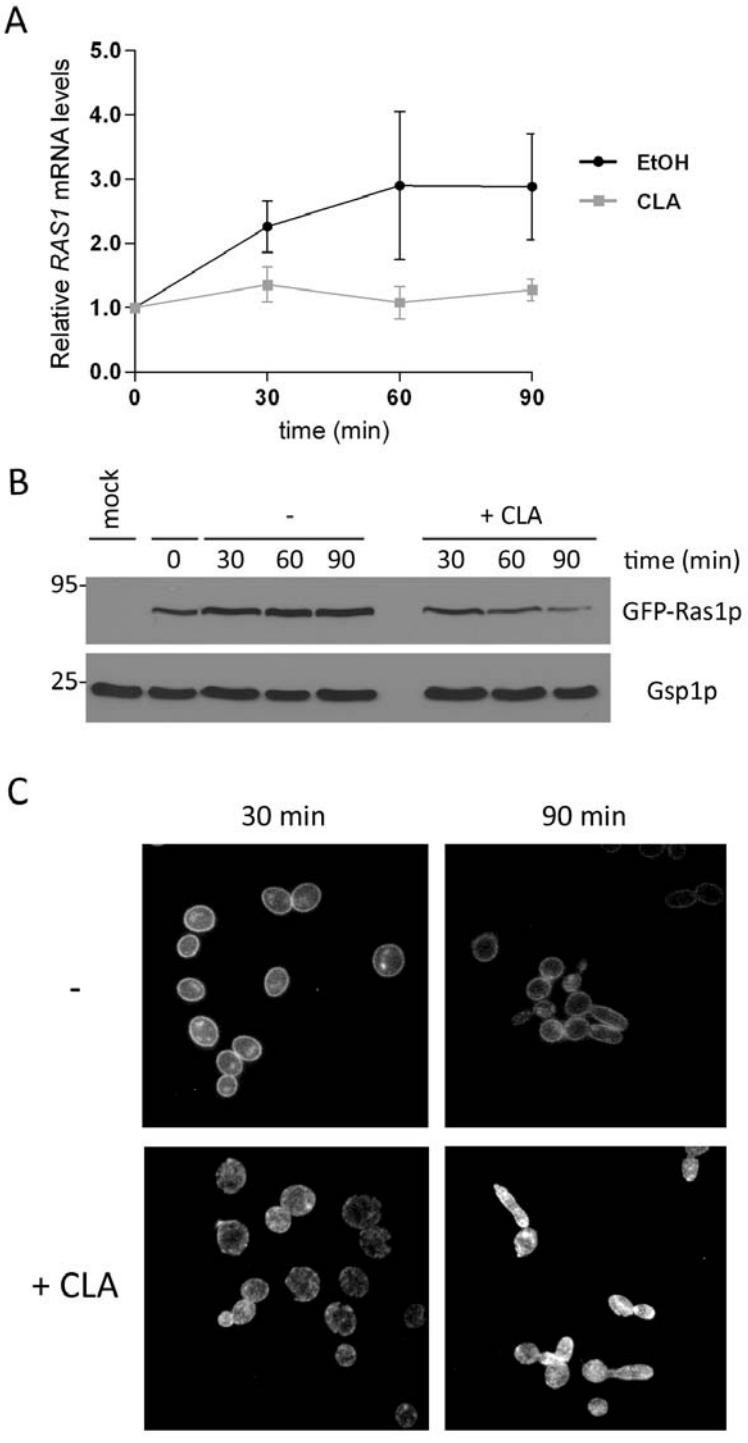
A
Relative RAS1 mRNA levels
5.0
4.0
3.0
2.0
1.0
0.0
0
30
60
90
time (min)
EtOH
CLA
B
mock
-
+ CLA
0
30
60
90
30
60
90
time (min)
95
GFP-Ras1p
25
Gsp1p
C
30 min
90 min
-
+ CLA

Figure 2. 6 CLA reduces GFP-Ras1p protein levels and affects its localization.

(A) SC5314 cells were grown in Spider medium at 37°C in the absence or presence of 25 μM CLA and harvested at the indicated time points. Transcript levels of *RAS1* were measured by quantitative PCR and normalized to those for *ACT1*. Relative expression levels were obtained by normalizing data for each time point to data obtained at time zero. Data are means and standard deviations for duplicate biological samples. (B) Ras1p protein levels were analyzed using a strain expressing GFP-Ras1p. Total protein extracts were prepared from SC5314 (mock) and GFP-Ras1p (WY-ZXD3) strains grown as described for panel A in the absence or presence of 100 μM CLA. Western blotting analysis was performed using anti-GFP antibodies. Gsp1p, shown as a loading control, was detected using antibodies raised against *S. cerevisiae* Gsp1p. Molecular masses (kDa) are indicated on the left. (C) GFP-Ras1p-expressing cells were grown in Spider medium at 30°C in the absence or presence of 100 μM CLA. Aliquots of cells were removed and examined directly at ×100 magnification using epifluorescence.

2.4.7 CLA affects the Tup1p-Nrg1p signaling pathway

CLA affects the Tup1p-Nrg1p signaling pathway. It has been established that the MAP kinase, Ras1p-cAMP-PKA, and Tup1p-Nrg1p signaling pathways make independent contributions to filamentation (Braun & Johnson, 2000). The hyphal growth repressor Tup1p functions with the DNA-binding proteins Nrg1p and Rfg1p to negatively regulate hyphal growth and hyphal gene expression (Braun & Johnson, 1997, Braun *et al*., 2001, Murad *et al*., 2001, Khalaf & Zitomer, 2001, Kadosh & Johnson, 2001). Given that Tup1p and Nrg1p are involved in mediating the inhibitory effect of farnesol on filamentation (Kebaara *et al*., 2008), their role in the CLA-mediated hyphal growth inhibition was then investigated. We examined the responses of *tup1/tup1* and *nrg1/nrg1* mutant strains to CLA when induced to filament in Spider medium at 37°C. While CLA inhibited hyphal growth in the parental strain CAI4, *tup1/tup1* and *nrg1/nrg1* mutant strains remained filamentous, even when higher CLA concentrations were used (Figure 2.7A). This indicates that CLA, like farnesol, inhibits filamentation by affecting a pathway requiring Tup1p and Nrg1p.

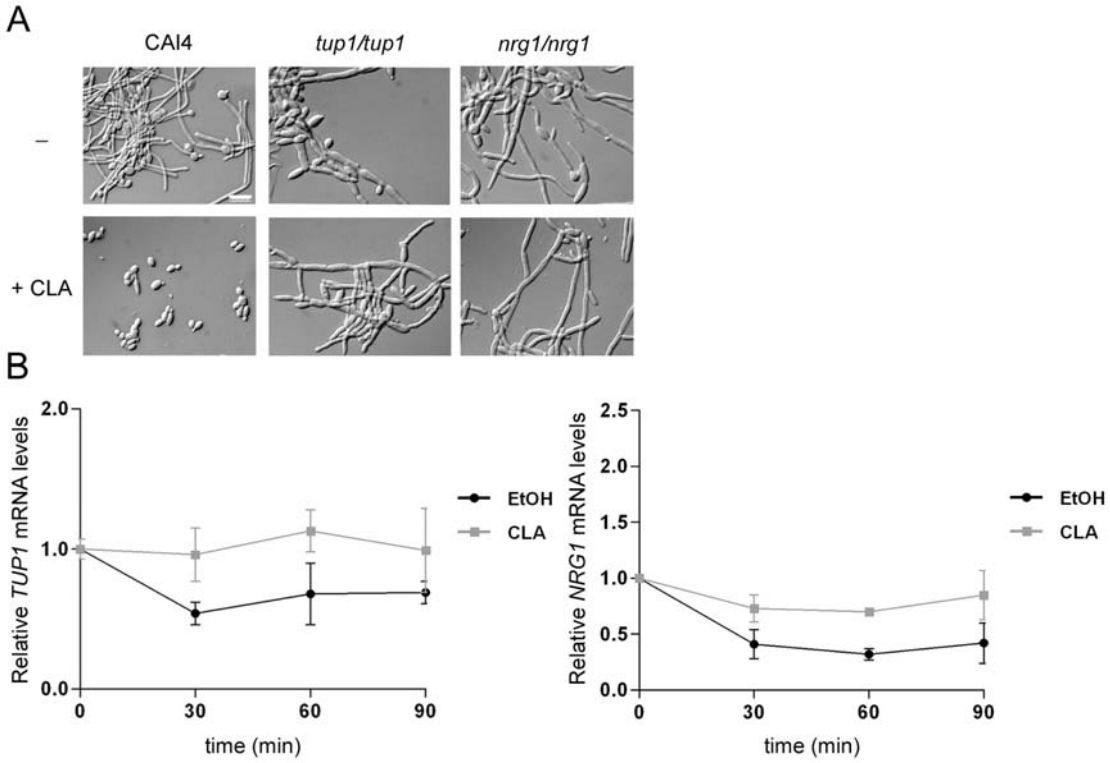

Figure 2. 7 CLA affects the Tup1p-Nrg1p signaling pathway.

(A) The CAI4, *tup1/tup1* (BCa2-10), and *nrg1/nrg1* (BCa23-3) strains were grown in Spider medium at 37°C in the absence or presence of 25 μM CLA for 4 h. Aliquots of cells were harvested and visualized at ×100 magnification by DIC optics. Bar = 10 μm. (B) *C. albicans* SC5314 cells were grown as described for panel A and harvested at the indicated time points. Transcript levels of *TUP1* and *NRG1* were measured as described for Figure 2.6A.

TUP1 and *NRG1* are modulated at the transcriptional level upon the yeast-to-hypha transition (Kebaara *et al*., 2008, Braun *et al*., 2001). According to transcriptional profiling, *TUP1* and *NRG1* were not significantly differentially expressed in response to CLA, but this could be due to their low expression levels. Thus, we assessed how CLA affected the kinetics of expression of *TUP1* and *NRG1*. Quantitative PCR revealed that *TUP1* and *NRG1* transcript levels decreased by ~2-fold during the 90-min time course in untreated

cells (Figure 2.7B). In contrast, CLA prevented the downregulation of both repressors, as *TUP1* and *NRG1* mRNA levels remained unchanged compared to initial levels. These findings suggest that CLA may inhibit hyphal growth by preventing the relief of repression exerted by the Tup1p-Nrg1p pathway.

2.5 Discussion

While efforts have been put forth to elucidate the molecular mechanisms underlying the yeast-to-hypha transition in *Candida albicans*, small molecules affecting the morphogenetic switch have been identified in concurrent studies. Our findings have enabled us to add CLA to the growing list of molecules that modulate hyphal growth, which includes farnesol, dodecanol, fatty acids and lipid metabolites, rapamycin, and geldanamycin as well as histone deacetylase inhibitors (Hornby *et al*., 2001, Davis-Hanna *et al*., 2008, Kebaara *et al*., 2008, Clement *et al*., 2007, Noverr & Huffnagle, 2004, Murzyn *et al*., 2010, Bastidas *et al*., 2009, Shapiro *et al*., 2009, Hnisz *et al*., 2010). CLA was effective at inhibiting hyphal growth in most hypha-inducing media (Figure 2.1). However, its inhibitory activity was significantly reduced in media containing FBS or N-acetylglucosamine, in RPMI 1640, or upon embedding of cells in YP medium (data not shown). The medium-dependent inhibitory effect of CLA on hyphal growth may be due to the nature or robustness of hypha-inducing signals, to the enhanced growth capacity of nutritionally rich media (Nantel *et al*., 2002, Toenjes *et al*., 2005), to the nonspecific lipid-binding capacity of serum albumin in FBS (Langford *et al*., 2009), or to the poor solubility of CLA in aqueous media (Ramirez & Lorenz, 2009).

Is CLA directly inhibiting filamentation or is this effect mediated by one of its metabolites? Fatty acids are nonfermentable carbon sources metabolized by *C. albicans* to acetyl-CoA and to glucose via β-oxidation, the glyoxylate cycle, and gluconeogenesis (Lorenz *et al*., 2004). Hallmark genes of these three biochemical pathways were upregulated in CLA-treated cells (see Table S2 to S4 in the supplemental material),

suggesting CLA was possibly being converted to glucose. We ruled out that fatty acid metabolism was involved in the CLA-mediated hyphal growth inhibition by confirming that CLA blocked filamentation of the *fox2/fox2*, *icl1/icl1*, and *ctf1/ctf1* mutant strains, which are unable to metabolize fatty acids (Ramirez & Lorenz, 2007, Ramirez & Lorenz, 2009) (data not shown). However, fatty acids are not only metabolized via β-oxidation, they can also be derived into oxygenated lipid metabolites. In *C. albicans*, arachidonic acid was shown to be a precursor for the production of 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18-diHETE), an eicosanoid found exclusively in hyphae which was suggested to play a role in morphogenesis (Deva *et al*., 2000). Fatty acids such as linoleic and arachidonic acids are also derived into prostaglandins and leukotrienes which reportedly affect hyphal development (Noverr *et al*., 2003, Erb-Downward & Noverr, 2007). Indeed, prostaglandin E2 and thromboxane B2 were shown to enhance the yeast-to-hypha transition (Kalo-Klein & Witkin, 1990, Noverr *et al*., 2001, Noverr & Huffnagle, 2004). Thus, while our results demonstrate that molecules derived from CLA's metabolism played no role in the CLA-mediated hyphal growth inhibition, we cannot exclude the possibility that oxygenated CLA derivatives may be involved in the effect.

We used global gene expression profiling as a means to investigate the transcriptional profile of cells induced to filament in presence of CLA. Temperature shift in Spider medium promotes a hyphal growth program similar to the one induced upon filamentation in Lee's medium (see Table S1 in the supplemental material) (Goyard *et al*., 2008). Both Spider and Lee's media have similar compositions, containing a source of fermentable carbon (mannitol or glucose), various amino acids, and salts, which may explain why both transcriptional profiles were similar. Transcript levels of hypha-specific genes, such as *ECE1*, *ALS3*, and *HYR1* and of several key regulators of hyphal growth, including *CPH1*, *CPH2*, *TEC1*, *BCR1*, *UME6*, *GAT2*, *RAS1*, and *CYR1*, increased during filamentation in Spider (Tables III and S1). However, the induction levels of several of these genes were lower than in other studies (Nantel *et al*., 2002, Goyard *et al*., 2008). This discrepancy may have been due to hypha-inducing signals being weaker in Spider medium

or to filamentation being somewhat induced in our control condition (Figure 2.3A). Nonetheless, the transcriptional data indicated roles for the Ras1p-cAMP-PKA, the MAP kinase, the *CPH2-TEC1* and the Tup1p-Nrg1p signaling pathways. These results confirmed that hyphal growth in Spider medium is regulated by a network of known signaling pathways which are activated simultaneously and converge (or do not converge) onto many of the same target transcription factors (Braun & Johnson, 2000, Biswas *et al*., 2007, Brown *et al*., 2007b).

Comparing the transcriptional profiles of untreated and CLA-treated cells revealed that CLA affected the expression of genes encoding signal transducers and transcription factors, including *RAS1*, *TEC1*, *UME6*, and *RFG1* (Figure 2.3C). We examined CLA's effect on hyphal growth in strains deleted for each of these genes and showed that *UME6* and *RFG1* were dispensable for CLA-mediated inhibition of filamentation (Figure 2.4). Similar conclusions for *RAS1* and *TEC1* could not be drawn, as both genes are required for hyphal growth in Spider medium (our observations and references (Leberer *et al*., 2001, Homann *et al*., 2009, Zhu *et al*., 2009). Given that *TEC1* lies downstream of the Ras1p-cAMP-PKA signaling pathway, we showed that CLA decreased *TEC1* expression levels in a mostly Ras1p-dependent manner (Figure 2.5B and C). Unexpectedly, while *TEC1* induction was partially Efg1p-dependent, CLA downregulated *TEC1* mRNA levels independently of *EFG1* (Figure 2.5B). These results demonstrated that CLA inhibited filamentation by affecting the Efg1p-independent branch of the Ras1p signaling pathway. Likewise, the Hsp90p inhibitor geldanamycin was shown to modulate hyphal growth by affecting the Ras1p-cAMP-PKA signaling pathway independently of Efg1p (Shapiro *et al*., 2009).

Transcriptional profiling further revealed that CLA affected *RAS1* expression (Figure 2.3C). Indeed, CLA blocked the increase in *RAS1* transcript levels which occurred upon the yeast-to-hypha transition (Figure 2.6A). Quite unexpectedly, while *RAS1* mRNA levels remained constant in CLA-treated cells, GFP-Ras1p levels declined gradually

(Figure 2.6B). CLA also affected localization of GFP-Ras1p to the plasma membrane (Figure 2.6C). Decreased GFP-Ras1p levels could be due to a decrease in its mRNA translation or to its degradation. However, several lines of evidence indicate that Ras delocalization results in its degradation and ultimately reduces its cellular levels. For instance, in *Saccharomyces cerevisiae*, a mutant Ras2p protein that could not be targeted to the membrane had lower cellular levels compared to the wild-type protein (Deschenes & Broach, 1987). Furthermore, farnesylthiosalicylic acid treatment was shown to dislodge Ras from the membrane and induce its degradation, resulting in lower Ras levels (Haklai *et al*., 1998). In light of our findings, we can only speculate that reduced GFP-Ras1p steady-state levels seen in CLA-treated cells stem from the protein being delocalized from the plasma membrane.

Several reasons may account for CLA's effect on GFP-Ras1p localization. First, the fatty acid may interfere with the posttranslational modifications of GFP-Ras1p. Ras proteins are modified by the addition of C-terminal lipids, such as farnesyl and palmitoyl moieties (Wennerberg *et al*., 2005). Such modifications are involved in membrane association and subcellular localization which are critical for Ras biological activities. Posttranslational modifications of Ras also play a role in filamentation in *C. albicans*, as compounds that prevent Ras prenylation were shown to inhibit hyphal growth (McGeady *et al*., 2002). Second, CLA may also affect GFP-Ras1p localization by modifying the lipid composition of membranes. By being incorporated into membrane phospholipids, unsaturated fatty acids such as CLA may alter membrane structure and function, influencing the interaction of resident proteins with the plasma membrane (Chapkin *et al*., 2008). For instance, the polyunsaturated fatty acid docosahexaenoic acid (DHA) was shown to decrease membrane association of Ras by weakening its interactions with phospholipid acyl chains (Collett *et al*., 2001, Seo *et al*., 2006). Interestingly, DHA also affected GFP-Ras1p localization to the plasma membrane in *C. albicans* (data not shown). The upregulation of *RTA3* transcript levels in CLA-treated cells may be genetic evidence that the fatty acid is modifying membrane lipid composition (see Table S2 to S4 in the

supplemental material). Indeed, the *S. cerevisiae RTA3* homologue (*RSB1*) was shown to be induced when plasma membrane glycerophospholipid asymmetry was altered (Ikeda *et al*., 2008). Nonetheless, further studies are warranted to address the underlying mechanism by which CLA exerts its effect on GFP-Ras1p membrane localization.

The biological activity of Ras is dictated by its subcellular localization. In *S. cerevisiae*, the farnesylated, membrane-bound form of Ras2p is approximately 100-times more effective in activating adenylate cyclase than its cytoplasmic form (Kuroda *et al*., 1993). Thus, it is tempting to suggest that CLA modulates the Ras1p signaling pathway by affecting the localization of Ras1p to the plasma membrane (Figure 2.6C) and by abrogating the increases in *RAS1* mRNA and protein levels (Figure 2.6A and B). CLA would prevent the activation of the Ras1p pathway, resulting in the downregulation of *TEC1* expression levels and inhibiting hyphal growth in *C. albicans* (Figure 2.8). Additionally, CLA may also inhibit hyphal growth by preventing the relief of repression exerted by the Tup1p-Nrg1p pathway (Figure 2.7B). Because the hyphal growth program is controlled by a network of signaling pathways, we expect the effect of CLA, like that of farnesol, to be multifactorial, in that the fatty acid could affect more than one signaling pathway (Langford *et al*., 2009). Nonetheless, our findings that CLA modulates *RAS1* mRNA and protein levels in *C. albicans* demonstrate the broad spectrum therapeutic properties of CLA, as the Ras pathway regulates filamentous growth and virulence in other fungi (D'Souza & Heitman, 2001).

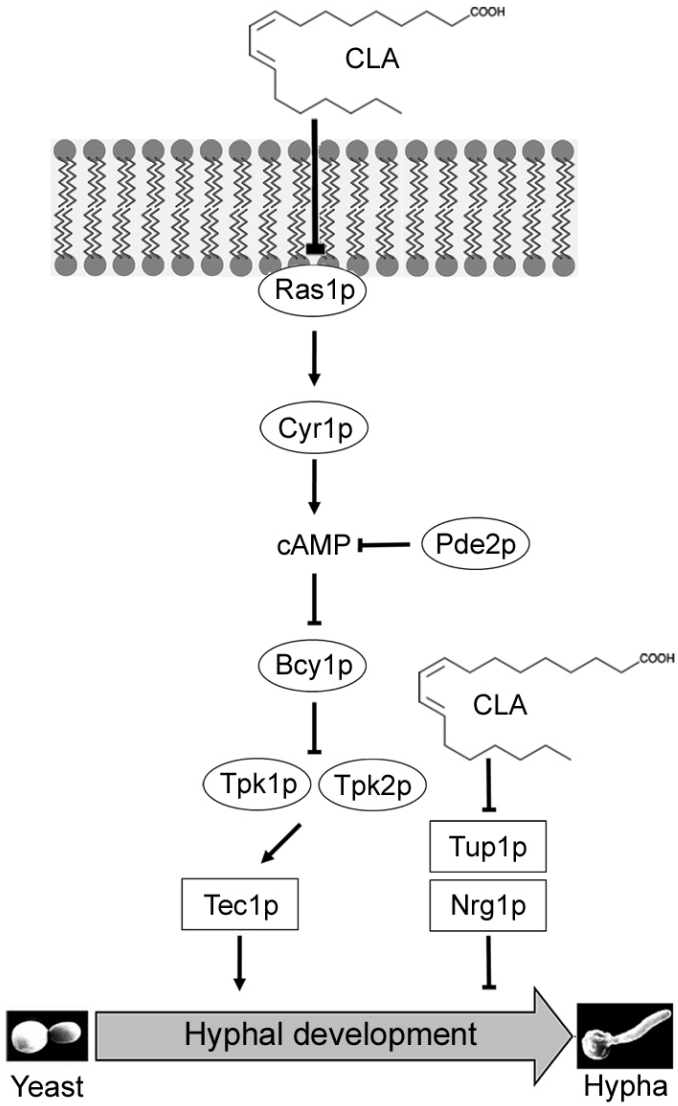

Figure 2. 8 Proposed model underlying the mechanism by which CLA inhibits hyphal growth in *Candida albicans*.

CLA inhibits the increase in *RAS1* mRNA and protein levels and affects Ras1p membrane localization. Combined, these CLA-mediated effects impede the activation of the Ras1 signaling pathway and ultimately downregulate *TEC1* expression and inhibit hyphal growth. In addition, CLA inhibits hyphal growth by preventing the relief of repression exerted by the Tup1p-Nrg1p complex.

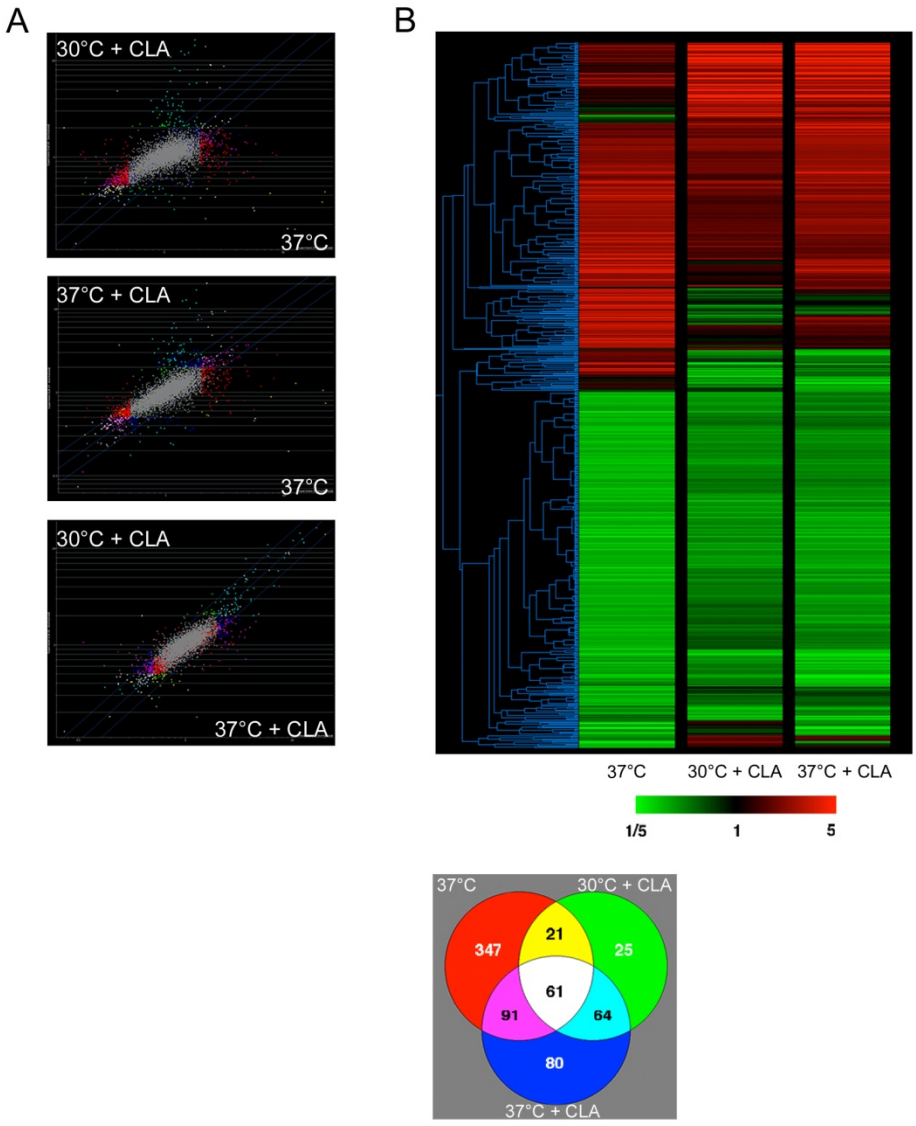

Figure S 1 Transcriptional profiles of CLA-treated cells.

(A) Scatter plots comparing pairs of transcriptional profiles. Genes with a statistically-significant change in transcript abundance are colored as indicated in the Venn diagram. (B) Hierarchical clustering of 689 genes with a statistically-significant change in transcript abundance (fold change > 2, p-value < 0.05) in cells grown at 37°C, or cells treated with CLA at 30°C or 37°C compared with control cells grown in Spider medium at 30°C. Also included are an additional 25 genes that show significant differences when pairs of profiles are directly compared to each other. Upregulated genes are colored in red while downregulated genes are colored in green.

2.6 Acknowledgments

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3. Chapter 3. Modulation of morphogenesis in *Candida albicans* by various small molecules

The yeast-to-hypha transition is a virulence factor in *Candida albicans*, and thus may constitute a target for the development of antifungal agents. A number of small molecules have been reported to modulate morphogenesis. These molecules are believed to harbor a therapeutic potential as they target a virulence trait of the pathogenic yeast. In this chapter, the literature pertaining to the modulation of morphogenesis by small molecules is reviewed; experimental findings regarding the modes of action and therapeutic properties of several small molecules are also addressed. After collating and examining data from relevant studies, we discuss whether or not modulating the yeast-to-hypha transition constitutes a strategy to treat *Candida* infections. The content of this chapter has been published in an article entitled "Modulation of morphogenesis in *Candida albicans* by various small molecules" in the scientific journal Eukaryotic Cell in 2011 (Shareck J. & Belhumeur P.).

Contribution: I chose to write a short review on this topic. I researched the literature, collated the data, and wrote the manuscript, which Belhumeur corrected. I estimate my contribution to being close to 95%.

Modulation of morphogenesis in *Candida albicans* by various small molecules

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3.1 Abstract

The pathogenic yeast *Candida albicans*, a member of the mucosal microbiota, is responsible for a large spectrum of infections, ranging from benign thrush and vulvovaginitis in both healthy and immunocompromised individuals to severe, life-threatening infections in immunocompromised patients. A striking feature of *C. albicans* is its ability to grow as budding yeast and as filamentous forms, including hyphae and pseudohyphae. The yeast-to-hypha transition contributes to the overall virulence of *C. albicans* and may even constitute a target for the development of antifungal drugs. Indeed, impairing morphogenesis in *C. albicans* has been shown to be a means to treat candidiasis. Additionally, a large number of small molecules such as farnesol, fatty acids, rapamycin, geldanamycin, histone deacetylase inhibitors, and cell cycle inhibitors have been reported to modulate the yeast-to-hypha transition in *C. albicans*. In this review, we take a look at molecules that modulate morphogenesis in this pathogenic yeast. When possible, we address experimental findings regarding their mechanisms of action and their therapeutic potential. We discuss whether or not modulating morphogenesis constitutes a strategy to treat *Candida* infections.

3.2 Introduction

Candida albicans, a member of the endogenous human microflora, is the most common human fungal pathogen. In healthy individuals, outgrowth of *C. albicans* results in superficial mycoses of the skin, nails, and mucous membranes (thrush and vulvovaginitis). However, in individuals with immune deficiencies caused by underlying disease, chemotherapy treatment, or immunosuppression following a transplantation, *C. albicans* can cause severe, life-threatening invasive candidiasis. *C. albicans* now ranks as the fourth leading cause of nosocomial infections and is the most common fungal species causing bloodstream infections, with associated mortality rates of 38 to 49% (Horn *et al.*, 2009; Miceli *et al.*, 2011; Pfaller & Diekema, 2007; Wisplinghoff *et al.*, 2004). Antifungal drugs

currently used for the treatment of *Candida* infections include polyenes, azoles, echinocandins, allyamines, and flucytosine. These drugs exert either fungicidal or fungistatic activities by interfering with essential processes (Odds *et al.*, 2003). Intensive prophylactic and therapeutic uses of antifungals have selected for drug-resistant strains (Anderson, 2005; Cowen *et al.*, 2002; Sanglard & White, 2007). Moreover, the limited arsenal of antifungal drugs is further compromised by severe side effects in patients and the emergence of species refractory to conventionally used agents (Miceli *et al.*, 2011). There is a need to develop new antifungals and to explore novel therapeutic approaches to treat *Candida* infections.

C. albicans has the ability to grow in a variety of morphological forms, including as budding yeast, pseudohyphae, and true hyphae (Sudbery *et al.*, 2004). The transition from yeast growth to hyphal growth is induced by a variety of environmental cues reflecting host conditions (temperature of 37°C, neutral or alkaline pH, or presence of serum) which activate a complex network of signaling pathways (Biswas *et al.*, 2007; Brown *et al.*, 2007; Ernst, 2000; Whiteway & Bachewich, 2007). Although recent findings have demonstrated that the yeast-to-hypha (Y-H) transition is not always required for virulence in systemic candidiasis (Noble *et al.*, 2010), morphogenesis still belongs to the realm of *C. albicans* virulence factors as demonstrated by several lines of evidence, the first being that strains defective in morphogenesis are attenuated in virulence in systemic candidiasis (Lo *et al.*, 1997; Saville *et al.*, 2003; Zheng & Wang, 2004). In addition, hyphal development is necessary for *C. albicans* to evade phagocytes (Lorenz *et al.*, 2004), to escape from blood vessels (Phan *et al.*, 2000), and to colonize medical devices by forming biofilms (Nobile & Mitchell, 2005; Nobile *et al.*, 2006). Moreover, both yeast and hyphal cells are found in *C. albicans*-infected organs (Odds, 1988). Thus, morphogenesis contributes to the overall virulence of *C. albicans*.

To widen the repertoire of antifungal drugs, targets that differ from those of conventional drugs have to be identified. Recently, targeting virulence rather than essential

processes has been postulated as a new paradigm for the development of antifungal agents, following the successful development of drugs targeting bacterial virulence in antimicrobial therapy (Alksne & Projan, 2000; Calugi *et al.*, 2011; Gauwerky *et al.*, 2009). Thus, instead of being killed, a pathogen is maintained in a harmless form by blocking virulence attributes that contribute to its pathogenicity. Moreover, resistance to drugs that target virulence instead of growth is less likely to develop, given that selective pressure is reduced on non-essential targets that are required only to colonize host environments (Jiang *et al.*, 2002). In *C. albicans*, virulence factors that are eligible as targets for the development of new antifungal drugs have been reviewed recently and include secreted aspartic proteases, phospholipases, calcineurin, inositol phosphoryl ceramide synthase, and elastase (Calugi *et al.*, 2011; Gauwerky *et al.*, 2009).

Given that morphogenesis is a virulence factor of *C. albicans*, it may also constitute a target for the development of antifungal drugs. Indeed, impairing morphogenesis has been shown to be a means to treat candidiasis. Using the *tet-NRG1* strain, which can be induced to filament when doxycycline is added to the drinking water of animals, studies have demonstrated that inhibiting filamentation attenuated virulence in a model of systemic candidiasis and served as an effective therapeutic intervention (Saville *et al.*, 2003; Saville *et al.*, 2006). Concurrently, numerous molecules have been reported to modulate the Y-H transition in *C. albicans*. These molecules may harbor interesting therapeutic properties, given that they target a virulence factor. In addition, they are in line with the current trend in antifungal drug development and may constitute novel antifungal agents, which are required to remedy antifungal drug resistance problems and to enhance the arnesal of antifungal agents. In this review, we research the literature in an attempt to list the molecules reported to influence morphogenesis in *C. albicans*, while focusing on studies that have addressed the modes of action of specific molecules. When possible, we review experimental findings regarding their effects on *C. albicans* infections. We discuss whether or not modulating morphogenesis constitutes a strategy to treat *Candida* infections.

3.3 Farnesol

Farnesol, a 15-carbon oxygenated lipid made up of isoprene moieties, was the first quorum sensing (QS) molecule to be identified in eukaryotes (Hornby *et al.*, 2001). Secreted by a number of *Candida* laboratory strains and clinical isolates, farnesol inhibits the Y-H transition in *C. albicans* (Hornby *et al.*, 2001; Hornby & Nickerson, 2004). The QS molecule is active at blocking hyphal growth induced by a variety of morphogenetic cues such as serum and N-acetylglucosamine (Mosel *et al.*, 2005). Moreover, farnesol affects various developmental processes in other *Candida* spp. and in pathogenic fungi including *Aspergillus* spp., *Fusarium graminearum*, and *Paracoccidiodes brasiliensis* (reviewed in Langford *et al.* 2009).

Findings regarding farnesol's repressive effects on filamentation and its mode of action have been reviewed extensively (Cottier & Muhlschlegel, 2009; Hogan, 2006; Kruppa, 2009; Langford *et al.*, 2009; Nickerson *et al.*, 2006). To gain insight into the response of *C. albicans* to farnesol, global gene expression analyses were performed (Cao *et al.*, 2005; Cho *et al.*, 2007; Enjalbert & Whiteway, 2005; Sato *et al.*, 2004). Although experimental approaches varied from one study to another, farnesol commonly affected the expression of genes that belonged to functional categories such as stress response, heat shock, drug resistance, amino acid and carbon metabolism, iron transport, cell wall, and cell cycle. One study suggested that farnesol affected the MAP kinase pathway, as transcript levels of the *HST7* kinase and the *CPH1* transcription factor were reduced in the presence of the molecule (Sato *et al.*, 2004). However, farnesol inhibited the Y-H transition in a *cph1/cph1* mutant, suggesting that *CPH1* is not a primary, but rather a secondary target of farnesol (Davis-Hanna *et al.*, 2008). While gene expression analyses generated a wealth of data pertaining to farnesol's transcriptional effects on *C. albicans*, specific cellular targets were not identified.

Other morphogenetic regulators may play a role in the *C. albicans* response to farnesol. *CHK1*, a two-component signal transduction pathway histidine kinase, appeared

to be required for the farnesol-mediated inhibition of hyphal growth (Kruppa *et al.*, 2004). Chk1p may act either as a sensor or downstream of the sensor for farnesol (Kruppa, 2009). The histidine kinase may function via the Hog1p MAP kinase, which was shown to be phosphorylated in the presence of farnesol (Smith *et al.*, 2004). Like *CHK1*, the negative regulators of filamentation *TUP1* and *NRG1* were shown to be involved in the response of cells to farnesol (Kebaara *et al.*, 2008). Indeed, *tup1/tup1* and *nrg1/nrg1* mutants remained filamentous in the presence of farnesol. Farnesol treatment also resulted in an increase in *TUP1* mRNA and protein levels and corrected the haploinsufficient phenotype of a *TUP1/tup1* mutant strain (Kebaara *et al.*, 2008). Concurrently, the Ras1p-cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway was identified as an important target of farnesol (Davis-Hanna *et al.*, 2008). Several lines of evidence suggest that farnesol inhibits the Y-H transition by downregulating Ras1p signaling. Farnesol repressed hypha formation in a strain that expressed the hyperactive Ras1p^{G13V} variant. Moreover, the addition of dibutyryl cAMP, a cAMP analogue, restored filamentation to farnesol-treated cells. Farnesol treatment also increased mRNA levels of cAMP-repressed genes, suggesting that cAMP levels were reduced in the presence of farnesol.

There are several ways by which farnesol could modulate the Ras1p signaling pathway as well as other factors involved in the Y-H transition. Membrane-bound, Ras1p interacts with the adenylate cyclase Cyr1p in the vicinity of the plasma membrane, thus promoting cAMP synthesis (Fang & Wang, 2006; Wennerberg *et al.*, 2005). Because farnesol is a highly hydrophobic molecule that localizes to the plasma membrane, it may disrupt the membrane, causing the release of farnesylated Ras1p or affecting the Ras1p-Cyr1p complex, thus impairing cAMP production and the Y-H transition (Davis-Hanna *et al.*, 2008; Langford *et al.*, 2009; Shchepin *et al.*, 2005). Farnesol may also impede posttranslational modifications of Ras1p, which are required for the protein's localization to the plasma membrane and its biological activity as well as for hyphal development in *C. albicans* (Hancock, 2003; Langford *et al.*, 2009; McGeady *et al.*, 2002). Another possibility is that farnesol binds directly to Ras1p or to Cyr1p, thus interfering with cAMP synthesis.

The effect of farnesol on *HST7* and *CPH1* expression levels (Sato *et al.*, 2004) is likely to be mediated by Ras1p, given that the GTPase is a regulator of the MAP kinase signaling pathway (Leberer *et al.*, 2001). Additionally, Tup1p/Nrg1p and Chk1p may also be downstream targets of Ras1p, as regulators of these factors have yet to be identified (Langford *et al.*, 2009). With the current state of knowledge regarding the molecular networks regulating the Y-H transition, it is impossible to determine whether farnesol has one specific target or modulates simultaneously different targets that vary according to hypha-inducing conditions. Furthermore, a receptor or a binding protein for farnesol has not been identified.

Given its hypha-inhibiting properties, farnesol was purported to harbor a therapeutic potential (Nickerson *et al.*, 2006) and its effects on various types of *Candida* infections were examined. *C. albicans* cells cocultured with human gingival fibroblasts and epithelial cells were exposed to farnesol. At concentrations tolerated by gingival cells, farnesol blocked the Y-H transition of *C. albicans* (Saidi *et al.*, 2006). In a follow-up study that used a human oral mucosa model, farnesol was shown to promote epithelial cell defense against *C. albicans* by increasing Toll-like receptor 2 (TLR2) expression levels, promoting interleukin-6 (IL-6) secretion, and increasing antimicrobial peptide human beta-defensin 2 (hBD2) levels (Decanis *et al.*, 2009). Likewise, using a mouse model of mucosal candidiasis, farnesol administered orally to infected animals suppressed hyphal development and had an overall protective effect against oral candidiasis (Hisajima *et al.*, 2008). Farnesol had contrasting effects in a mouse model of systemic candidiasis. Whether administered intraperitoneally, intravenously, or orally to infected mice, farnesol treatment accelerated the demise of infected animals (Navarathna *et al.*, 2007a). Moreover, farnesol interfered with the normal progression of cytokine induction by decreasing IL-12 levels, which increased the susceptibility of mice to systemic candidiasis (Navarathna *et al.*, 2007b). Administered on its own, farnesol was harmless, indicating it specifically enhanced virulence in systemic candidiasis. Taken together, these findings suggest that farnesol has

therapeutic potential in the context of oral mucosal infections but cannot be used to treat systemic candidiasis.

3.4 Bacterial and fungal autoregulatory molecules

In addition to farnesol, *C. albicans* secretes other autoregulatory molecules which influence the Y-H transition (Hogan, 2006; Kruppa, 2009). The fusel alcohols 2-phenylethanol and tryptophol were the first molecules reported to inhibit hyphal growth (Lingappa *et al.*, 1969). Yet, these molecules are not QS molecules, given that they are inhibitory at concentrations the yeast cannot produce naturally (Hazen & Cutler, 1979). An unknown substance termed MARS (morphogenic autoregulatory substance), isoamyl alcohol, and E-nerolidol have also been shown to possess hypha-inhibiting activities (Hazen & Cutler, 1979; Hazen & Cutler, 1983; Martins *et al.*, 2007). Farnesoic acid, which is structurally related to farnesol, also blocked the Y-H transition (Oh *et al.*, 2001). However, it only possessed 3% of farnesol's inhibitory activity when germ tube formation was induced in N-acetylglucosamine-containing medium (Shchepin *et al.*, 2003). *PHO81*, which encodes a phosphatase, was recently shown to be required for farnesoic acid to inhibit the Y-H transition (Chung *et al.*, 2005; Chung *et al.*, 2010). Additionally, *PHO81* was proposed to be a negative regulator of Ras1p activity, as it appears to act upstream of Ras1p signaling (Chung *et al.*, 2010). In contrast to these hypha-inhibiting molecules, tyrosol was described as a QS molecule that stimulated germ tube formation (Chen *et al.*, 2004) and promoted hyphal development in the early stages of biofilm formation (Alem *et al.*, 2006). Tyrosol also reduced the lag phase of growth in a diluted culture, which may account for its hypha-stimulating properties (Chen *et al.*, 2004).

As a commensal of the mucosal microbiota and a pathogen in different host niches, *C. albicans* encounters microorganisms of the endogenous microflora as well as several opportunistic pathogens. A number of bacteria and yeast have been reported to secrete molecules that influence the Y-H transition. *Pseudomonas aeruginosa* secretes the 3-oxo-

C12-acyl homoserine lactone (3OC12HSL), a 12-carbon backbone molecule structurally related to farnesol, which inhibited the Y-H transition induced in N-acetylglucosamine-containing medium and caused filaments to revert to the yeast morphology (Hogan *et al.*, 2004). Similar in structure to farnesol and farnesoic acid, dodecanol and three other QS molecules produced by *Xanthomonas campestris*, *Burkholderia cenocepacia*, and *Streptococcus mutans* also exerted hypha-inhibiting activities (Boon *et al.*, 2008; Vilchez *et al.*, 2010; Wang *et al.*, 2004). Like farnesol, these molecules may block the Y-H transition by inhibiting the Ras1p-cAMP-PKA signaling pathway, potentially through similar mechanisms (Hogan, 2006; Hogan & Sundstrom, 2009). Additionally, *P. aeruginosa* also had an inhibitory effect on *C. albicans* growth in vitro, in burn wounds, and in the lungs of patients with cystic fibrosis (Gupta *et al.*, 2005; Kerr, 1994). Pyocyanin, phospholipase C, and phenazines were the molecules responsible for this inhibitory effect (Hogan & Kolter, 2002; Kerr *et al.*, 1999). Interestingly, the antifungal activity of *P. aeruginosa* was specifically targeted towards hyphal cells, as bacteria were shown to attach to and kill fungal filaments only (Hogan & Kolter, 2002).

Other microorganisms, including oral and gut residents, have also been reported to exert morphogenesis-modulating activities. *S. mutans* secreted a 22-amino acid-containing peptide which inhibited the Y-H transition induced using saliva-coated culture plates containing YNB medium (Jarosz *et al.*, 2009). In contrast, cell-free *S. gordonii* supernatants enhanced hyphal development and biofilm formation induced in presence of human saliva at 37°C and affected the activation of several MAP kinases, including that of Cek1p, Mkc1p, and Hog1p (Bamford *et al.*, 2009). Although the active compound was not identified, H_2O_2 and autoinducer 2 were proposed to promote filamentation by impacting the oxidative stress response. Culture supernatants from *Lactobacillus rhamnosus* GG and the probiotic yeast *Saccharomyces boulardii* blocked germ tube formation induced in serum-containing and in RPMI media at 37°C (Krasowska *et al.*, 2009; Murzyn *et al.*, 2010; Noverr & Huffnagle, 2004). Butyric acid was shown to mimic the effect of *L. rhamnosus* GG spent medium, at concentrations well within the physiological range

observed in the colon (Noverr & Huffnagle, 2004; Saemann *et al.*, 2002). The hypha-inhibiting activities of butyric acid had previously been reported (Braun *et al.*, 1987; Hoberg *et al.*, 1983). In the case of *S. boulardii*, capric acid was identified as the active molecule: it inhibited the Y-H transition as well as adhesion and biofilm formation (Murzyn *et al.*, 2010). Two other compounds produced by *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhimurium also inhibited filamentation and biofilm formation but have yet to be identified (Peleg *et al.*, 2008; Tampakakis *et al.*, 2009).

In complex microbial communities, molecules mediate interspecies interactions (Hogan, 2006; Hogan & Kolter, 2007; Wargo & Hogan, 2006). With respect to morphogenesis-modulating molecules, these secreted products may be a mechanism by which microbes compete with *C. albicans* by inhibiting hypha formation, limiting attachment, and preventing invasion. It may also be a way for *C. albicans* to respond to the presence of microorganisms. For instance, in presence of 3OC12HSL, *C. albicans* may block hyphal development as a means to escape being killed by *P. aeruginosa*. Conversely, hypha-inducing molecules may mediate synergistic interactions between bacteria and *C. albicans* by promoting biofilm formation and invasion (Bamford *et al.*, 2009).

3.5 Fatty acids, eicosanoids, and cyclooxygenase inhibitors

Lipids are involved in fungal development and pathogenicity (Erb-Downward & Huffnagle, 2006; Noverr *et al.*, 2003; Rhome & Del Poeta, 2009; Shea & Del Poeta, 2006). In *C. albicans*, lipid molecules such as fatty acids and eicosanoids have been reported to modulate the Y-H transition. Fatty acids, including butyric, capric, lauric, palmitoleic, oleic, linoleic, conjugated linoleic, and arachidonic acids, inhibited the Y-H transition induced in various conditions (Clement *et al.*, 2007; McLain *et al.*, 2000; Murzyn *et al.*, 2010; Noverr & Huffnagle, 2004). The hypha-inhibiting activities of fatty acids were dependent on the medium. For instance, linoleic and oleic acids were shown to have no effect on germ tube formation induced in serum or to block the Y-H transition induced in

other conditions, such as Spider medium (Clement *et al.*, 2007; Noverr & Huffnagle, 2004). Such discrepancies may be due to the lipid-binding capacity of serum albumins, which sequester lipidic molecules and reduce their effective concentrations (Langford *et al.*, 2009). Moreover, although the effects of fatty acids on the Y-H transition have been described, their modes of action have not been studied extensively. Conjugated linoleic acid (CLA) was reported to block the Y-H transition induced in Spider medium by affecting the subcellular localization of Ras1p and reducing its levels, thereby impeding the activation of Ras1p signaling and blocking the induction of the *TEC1* transcription factor (Shareck *et al.*, 2011). In addition, while the antifungal properties and cytotoxicity of several fatty acids have been reported (Bergsson *et al.*, 2001; Carballeira, 2008; Deva *et al.*, 2000), fatty acids inhibited hyphal growth at concentrations that did not affect cellular growth (Shareck *et al.*, 2011).

In contrast to fatty acids, eicosanoids such as prostaglandin E2 (Erb-Downward & Noverr, 2007; Kalo-Klein & Witkin, 1990; Noverr *et al.*, 2001) and thromboxane B2 (Noverr & Huffnagle, 2004) enhanced the Y-H transition. Eicosanoids are oxygenated lipids derived from 20-carbon polyunsaturated fatty acid precursors such as arachidonic acid. In mammalian cells, cyclooxygenases (COX) catalyze the conversion of arachidonic acid into prostaglandins. While *C. albicans* does not possess a COX homolog, the fatty acid desaturase *OLE2* and the multicopper oxidase *FET3* were found to play a role in prostaglandin synthesis (Erb-Downward & Noverr, 2007). Concurrently, several COX inhibitors, including diclofenac sodium, indomethacin, ibuprofen, resveratrol, and eicosatetraynoic acid (ETYA), were shown to block the Y-H transition induced in serum-containing, Lee's or Spider media at 37°C (Alem & Douglas, 2004; Ghalehnoo *et al.*, 2010; Okamoto-Shibayama *et al.*, 2010; Toenjes *et al.*, 2009). It is not clear whether or not COX inhibitors affected filamentation by blocking prostaglandin synthesis, given that a direct association between reduced prostaglandin levels and reduced hypha formation was not shown and that *C. albicans* does not encode a COX homolog. Moreover, COX inhibitors appeared to reduce viability of *C. albicans* cells, which may account for their hypha-

inhibiting activities (Alem & Douglas, 2004; Deva *et al.*, 2001; Noverr *et al.*, 2001). For instance, diclofenac sodium and resveratrol blocked hyphal development but also affected cellular growth (Ghalehnoo *et al.*, 2010; Okamoto-Shibayama *et al.*, 2010).

3.6 Peptides and proteins

Various peptides and proteins were shown to modulate the Y-H transition in *C. albicans*. At concentrations of 25 μg ml-1, nisin Z, an antimicrobial peptide of the lantibiotic family, reduced germ tube formation induced in serum-containing medium and decreased adhesion of *C. albicans* cells to gingival monolayer cultures (Akerey *et al.*, 2009). However, a previous study had shown that at slightly higher concentrations (100 μg ml-1), nisin Z reduced cellular growth rates and caused ultrastructural disturbances in *C. albicans* cells, casting doubt on the mechanism by which the antimicrobial peptide inhibited filamentation (Le Lay *et al.*, 2008). Salivary components statherin and mucin also affected morphogenesis. Hyphae grown overnight in RPMI medium switched to yeast growth in presence of statherin (Leito *et al.*, 2009), while mucin inhibited hypha formation induced in RPMI medium at 37°C and blocked *RAS1* induction without affecting cellular growth (Ogasawara *et al.*, 2007). *C*dc42/*R*ac *i*nteractive *b*inding (CRIB) fusion polypeptides containing the CRIB consensus sequence were designed to disrupt the binding of the Rho GTPase Cdc42p to its effectors Cst20p and Cla4p, interactions that are required for hyphal development (Leberer *et al.*, 1996; Leberer *et al.*, 1997). CRIB polypeptides, which have high-affinity binding to Cdc42p, blocked the Y-H transition induced in Lee's medium in time- and dose-dependent fashions, without impairing cellular growth (Su *et al.*, 2007). Additionally, hyphal cells grown overnight in Lee's medium switched to yeast-like forms when they were incubated in fresh medium in the presence of either of the CRIB polypeptides, indicating the peptides can convert hyphal cells back into the yeast-like form.

3.7 Rapamycin

Rapamycin, a hydrophobic macrolide produced as a secondary metabolite by the soil bacterium *Streptomyces hygroscopicus*, was initially discovered as an antifungal agent against *C. albicans* (Vezina *et al.*, 1975). Rapamycin inhibits the function of the Tor (target of rapamycin) kinases (Heitman *et al.*, 1991), of which one homolog was identified in *C. albicans* (Tor1p) (Cruz *et al.*, 2001). Tor1p has contrasting roles in regulating morphogenesis which vary according to hypha-inducing conditions. For instance, on solid synthetic low ammonium dextrose (SLAD) medium, alkaline M199 medium (pH 8.0), and Spider medium at 37°C, sublethal concentrations of rapamycin blocked hyphal growth, indicating that Tor1p is a positive regulator of filamentation under nitrogen or nutrient starvation conditions and in response to alkaline growth conditions (Bastidas *et al.*, 2009; Cutler *et al.*, 2001; Martins *et al.*, 2008). In contrast, in most liquid hypha-inducing media, rapamycin had no effect on the Y-H transition, indicating that Tor1p does not regulate hyphal development under these conditions (Bastidas *et al.*, 2009).

Interestingly, in liquid Spider medium, rapamycin promoted cellular aggregation and flocculation (Bastidas *et al.*, 2009). These findings demonstrate that Tor1p negatively regulates cellular adhesion under such growth conditions. Gene expression analysis showed that rapamycin treatment strongly induced the hyphal growth program in *C. albicans*, resulting in the induction of hypha-specific genes (*ALS1*, *ALS3*, *HWP1*, and *ECE1*) and hyphal growth regulators (*TEC1* and *RFG1*). Additionally, rapamycin decreased transcript levels of filamentation repressors *TUP1* and *NRG1*. The transcription factors Efg1p and Bcr1p were required for the rapamycin-mediated cellular aggregation as well as for expression of adhesins. Thus, in liquid Spider medium, rapamycin promotes cellular aggregation by compromising Tor1p function, which results in the activation of Efg1p and Bcr1p and the downregulation of *TUP1* and *NRG1*.

3.8 Geldanamycin

Geldanamycin (GdA) is a microbial metabolite that inhibits the function of Hsp90p, a molecular chaperone and heat-shock protein (Roe *et al.*, 1999). Hsp90p regulates the form and function of various client proteins, several of which regulate morphogenesis in *C. albicans* (Pearl & Prodromou, 2006; Pratt & Toft, 2003; Shapiro *et al.*, 2009). GdA treatment induced filamentation in *C. albicans* cells grown in noninducing conditions (Shapiro *et al.*, 2009). The genetic depletion of *HSP90* was also shown to phenocopy the GdA-induced filamentation. Combined, these results suggest that Hsp90p is a negative regulator of morphogenesis. Upstream inputs from the cAMP-PKA signaling pathway were required for cells to filament in presence of GdA, while the transcription factor Efg1p was not required. These results suggest that the GdA-induced filamentation may be regulated by transcription factors that function downstream of PKA, such as Tec1p, Flo8p, and Sfl1p (Shapiro *et al.*, 2009; Shapiro & Cowen, 2010). Hsp90p has been proposed to repress cAMP-PKA signaling, either by (i) interacting with a positive regulator of the pathway, and maintaining it in an inactive form (e.g., Tpk1p or Tpk2p, the catalytic subunits of PKA), (ii) stabilizing a negative regulator of the pathway (e.g., Bcy1p, the regulatory subunit of PKA), or (iii) interacting indirectly with the cAMP-PKA pathway via an unknown protein (Shapiro & Cowen, 2010). Other models involving different Hsp90p client proteins are also possible. Interestingly, the genetic depletion of *HSP90* attenuated virulence in a mouse model of systemic candidiasis, resulting in clearance of the infection (Shapiro *et al.*, 2009). While these findings suggest that modulating morphogenesis by compromising Hsp90p function is a means to treat candidiasis, reduced virulence may have been due to reduced cellular growth rates associated with reduced HSP90 cellular levels. Nonetheless, a study has shown that harnessing Hsp90p function, either with the use of clinically relevant GdA derivatives or by genetically compromising *HSP90*, improved the therapeutic efficacy of an azole in two animal models of infection (Cowen *et al.*, 2009). Because they enhance the efficacy of existing antifungal drugs, Hsp90p inhibitors such as GdA and structurally related compounds are good candidates for the development of effective combination therapy strategies.

3.9 Histone deacetylase inhibitors

Histone deacetylases (HDACs) are chromatin-remodeling proteins usually involved in transcriptional repression (Grozinger & Schreiber, 2002). HDACs deacetylate histones globally or at specific promoters, directed by transcription factors or other DNA-binding proteins (Kadosh & Struhl, 1998). The HDACs Hda1p, Hos2p, Set3p, and Hst3p have been reported to regulate morphogenesis, yet they have contrasting roles (Hnisz *et al.*, 2010; Wurtele *et al.*, 2010; Zacchi *et al.*, 2010). Moreover, several HDAC inhibitors have been shown to modulate hyphal development and virulence (Hnisz *et al.*, 2010; Simonetti *et al.*, 2007; Smith & Edlind, 2002; Wurtele *et al.*, 2010). For instance, suberoylanilide hydroxamic acid (SAHA) inhibited the serum-induced Y-H transition, which correlated with a minor reduction in *EFG1* transcript levels (Simonetti *et al.*, 2007). In contrast, nicotinamide, an inhibitor of Hst3p, induced filamentation of *C. albicans* cells in noninducing conditions (Wurtele *et al.*, 2010). Hst3p, a member of a family of NAD^+-dependent histone deacetylases known as sirtuins, is inhibited by nicotinamide, a product of the NAD^+-dependent deacetylation reaction. The pharmacological inhibition of Hst3p using nicotinamide or the genetic depletion of *HST3* not only induced filamentation but also attenuated virulence in a model of systemic candidiasis. Yet, reduced virulence in vivo was likely due to reduced growth rates rather than to the modulation of morphology, as nicotinamide treatment or the repression of *HST3* resulted in cell death.

Trichostatin A (TSA), a well-characterized HDAC inhibitor (Yoshida *et al.*, 1995), had contrasting effects on morphogenesis that depended on experimental settings, including strains, growth conditions, and TSA concentrations used. Clinical isolates of *C. albicans* pretreated for 48 h with 4 μg ml^{-1} of TSA were unable to form germ tubes when induced to filament in yeast extract-peptone-dextrose (YPD) medium supplemented with 20% serum (Simonetti *et al.*, 2007). In addition, *EFG1* transcript levels were reduced in TSA-treated cells. These results suggest that TSA inhibited a histone deacetylase required for the Y-H transition and downregulated *EFG1* expression. The Hda1p HDAC was proposed to potentially mediate the effects of TSA on hyphal development. In contrast, similar TSA

concentrations had no effect on the serum-induced Y-H transition (Smith & Edlind, 2002). In these experiments, TSA treatment may have been insufficient to affect morphogenesis, given that the HDAC inhibitor was added at the onset of hyphal induction. Another study showed that TSA treatment promoted hyphal development under noninducing conditions (Hnisz *et al.*, 2010). Indeed, on solid YPD medium supplemented with 10 μg ml^{-1} of TSA, colonies displayed filamentous growth after 3 days of incubation at 37°C. The TSA-induced filamentation was phenocopied by the deletion of the *SET3* and *HOS2* histone deacetylase genes. Additionally, both histone deacetylases were shown to be involved in repressing morphogenesis at the level of the Efg1p transcription factor. Thus, the TSA-induced filamentation appears to be caused by TSA inhibiting the Set3p and Hos2p HDACs and relieving the repression of filamentation (Hnisz *et al.*, 2010). Interestingly, despite the fact the *set3/set3* mutant strain filamented in vivo, it was still attenuated in virulence in a model of systemic candidiasis. Moreover, reduced growth rates did not account for reduced virulence, as generation times of wild-type and *set3/set3* strains did not differ significantly (Hnisz *et al.*, 2010). Given that TSA inhibits Set3p, the HDAC inhibitor may reduce virulence in vivo, but this has yet to be demonstrated.

3.10 Cell cycle inhibitors

Hydroxyurea (HU) and nocodazole (NZ) are cell cycle-perturbing agents. While HU inhibits ribonucleotide reductase, depleting ribonucleotides and inhibiting DNA synthesis, NZ disrupts microtubules and locks cells in mitosis. In addition to causing cell cycle arrest, both drugs also trigger hyperpolarized growth of *C. albicans* cells grown in noninducing conditions (Bachewich *et al.*, 2003; Bachewich *et al.*, 2005; Bai *et al.*, 2002; Whiteway & Bachewich, 2007). Upon exposure to HU or to NZ, yeast cells developed hyperpolarized buds, which continued to elongate despite DNA replication being blocked (Bachewich *et al.*, 2003; Bachewich *et al.*, 2005; Bai *et al.*, 2002). HU- and NZ-induced elongated buds displayed pseudohyphal morphological features (constrictions at the neck and width of >2 μm) as well as hyphal-like features, in that they maintained polarized growth, demonstrated

nuclear movement out of the mother cell and into the elongating filament, and expressed several hypha-specific genes (HSGs) (Bachewich *et al.*, 2003; Bachewich *et al.*, 2005). HU-induced elongated buds eventually died after 24 h. However, sublethal concentrations of the inhibitor have also been reported to induce hyperpolarized growth without impacting cellular growth (Shi *et al.*, 2007). The development of hyperpolarized buds involves different proteins, several of which are cell cycle checkpoints. For instance, the Rad53p effector kinase of the DNA replication checkpoint and the Mad2p spindle assembly checkpoint were required for hyperpolarization of buds induced by HU and NZ treatments, respectively (Bai *et al.*, 2002; Shi *et al.*, 2007). Interestingly, bud elongation appears to be caused by the activation of cell cycle checkpoints by cell cycle inhibitors rather than by cell cycle arrest (Shi *et al.*, 2007). Moreover, several components of Ras1p signaling, including the GTPase Ras1p and adenylate cyclase Cyr1p, were required for HU-induced elongated buds (Bachewich *et al.*, 2003; Bachewich *et al.*, 2005). Given that hyperpolarized growth has been observed only in response to cell cycle-inhibiting agents or in strains in which cell cycle genes are inactivated or overexpressed (reviewed in Berman, 2006; Whiteway & Bachewich, 2007), it is not clear whether or not this growth mode is physiologically relevant. Yet, it remains that hyperpolarized buds may be important for pathogenicity and/or survival in the host, as they eventually express hypha- and virulence-specific factors (Whiteway & Bachewich, 2007).

3.11 Other small molecules

Many other small molecules have been reported to affect morphogenesis in *C. albicans* (Toenjes *et al.*, 2005; Toenjes *et al.*, 2009). Propranolol, a calmodulin inhibitor (Volpi *et al.*, 1981), was shown to inhibit serum-induced hypha formation by reducing *EFG1* expression levels (Baker *et al.*, 2002; Ueno *et al.*, 2009). Various inhibitors of actin dynamics, including latrunculin, jasplakinolide, and cytochalasin A, also blocked the Y-H transition induced in serum-containing, Spider or M199 media at 37°C (Akashi *et al.*, 1994; Hazan & Liu, 2002; Toenjes *et al.*, 2005; Wolyniak & Sundstrom, 2007). 1,4-Diamino-2-

butanone (DAB) blocked the Y-H transition induced in RPMI medium at 37°C by reducing polyamine levels, decreasing *CYR1* mRNA levels, and reducing cAMP cellular levels (Ueno *et al.*, 2004). Sublethal concentrations of azoles inhibited hypha formation induced in M199 medium at 37°C (Ha & White, 1999; Odds *et al.*, 1985). Azoles' hypha-inhibiting activities may be due to increased farnesol production reported in azole-treated cells (Hornby & Nickerson, 2004). Two derivatives of propanol from a chemical library reduced *C. albicans*-induced endothelial injury, most likely by preventing hypha formation (Toenjes *et al.*, 2005). The cellular targets of these structurally different molecules are not known. In contrast, hydrogen peroxide and the iron chelator bathophenanthrolene disulfonic acid (BPS) both induced hyphal development in noninducing conditions (Hameed *et al.*, 2008; Nasution *et al.*, 2008). BPS promoted filamentation by increasing *EFG1* mRNA levels.

Additionally, several cytostatic or cytotoxic molecules have been reported to inhibit the Y-H transition in *C. albicans*, including garlic extracts (Low *et al.*, 2008), a Mannich ketone compound (Kocsis *et al.*, 2009), the monoterpene thymol (Braga *et al.*, 2007), riccardin D, a macrocyclic bisbibenzyl compound isolated from Chinese liverwort *Dumortia hirsute* (Cheng *et al.*, 2009), and ECC145 and ECC188, two compounds that impair the fatty acid Δ9 desaturase Ole1p and block the biosynthesis of unsaturated fatty acids (Xu *et al.*, 2009). Lithium affected filamentation on solid galactose- and serum-containing media, but also reduced cellular growth in presence of galactose (Martins *et al.*, 2008). However, it is unclear whether or not lithium impacted growth rates in the presence of serum. It remains possible that molecules that are toxic to cells cannot be considered to have hypha-inhibiting properties, given that they might impede the Y-H transition merely by impairing cellular growth.

3.12 Conclusion

Many small molecules have been reported to modulate morphogenesis in *C. albicans*. Yet, an overview of the literature revealed that the mechanisms of few of these

compounds have been investigated. Interestingly, studies that have addressed the modes of action of small molecules have shown that many of these compounds target components of the Ras1p signaling pathway (Figure 3.1). It is surprising that several structurally different molecules commonly affect *RAS1*, *CYR1*, *EFG1*, *HST7* and *CPH1* mRNA levels, modulate cAMP levels or Efg1p-dependent transcription, or require components of the pathway to exert their morphogenesis-modulating activities (Table V). On one hand, despite being structurally different, it may be expected that these molecules modulate hyphal growth by targeting components of the Ras1p signaling pathway, given the major role this signaling pathway plays in morphogenesis. On the other hand, it is also possible that these common components are not primary targets, but rather secondary targets of various molecules. For instance, while farnesol was reported to downregulate *CPH1* transcript levels (Sato *et al.*, 2004), the transcription factor was not required for the QS molecule to block the Y-H transition (Davis-Hanna *et al.*, 2008). These results suggest that farnesol's effect on *CPH1* expression levels was a consequence of farnesol affecting its primary target, i.e. the Ras1p-cAMP-PKA signaling pathway. Likewise, components of the Ras1p signaling pathway that have been identified as primary targets of morphogenesis-modulating molecules may well be secondary targets.

Proof-of-concept experiments have shown that interfering with the morphogenetic plasticity of *C. albicans* and promoting its growth in a form that does not damage the host attenuates virulence in vivo (Saville *et al.*, 2003; Saville *et al.*, 2006). Therefore, does modulating morphogenesis constitute a sound approach to treat candidiasis? First, it is noteworthy to mention that there is an overall lack of evidence demonstrating that impairing the developmental process can be used to treat candidiasis. While many small molecules have been reported to modulate the Y-H transition in vitro, very few compounds have been evaluated for their effects on virulence using in vitro or in vivo infection models. Second, the therapeutic potential of some molecules, including nicotinamide, ECC145, ECC188, and geldanamycin, may stem in part from their cytotoxicity toward *C. albicans* rather than from their capacity to modulate the Y-H transition, as they target essential gene

products. Indeed, the genetic depletion of *HST3*, *OLE1*, and *HSP90* modulated morphogenesis in *C. albicans* and attenuated virulence in a model of systemic candidiasis but also reduced cellular growth rates (Shapiro *et al.*, 2009; Wurtele *et al.*, 2010; Xu *et al.*, 2009). Third, the studies that have evaluated the effects of small molecules on virulence in various infection models have yielded mixed results. Farnesol and nisin Z were both shown to have a protective effect in mucosal candidiasis (Akerey *et al.*, 2009; Hisajima *et al.*, 2008; Saidi *et al.*, 2006), while in a model of systemic candidiasis, farnesol enhanced pathogenicity (Navarathna *et al.*, 2007a; Navarathna *et al.*, 2007b).

On the basis of current findings, it appears that modulating morphogenesis is a means to treat mucosal/superficial *Candida* infections. As for systemic candidiasis, the lack of literature renders it difficult to reach similar conclusions. To circumvent this impediment, small molecules that modulate the Y-H transition in *C. albicans* without affecting cellular growth should be evaluated for their therapeutic potential in various infection models. More data are needed to determine whether or not targeting the Y-H transition constitutes a sound therapeutic strategy to treat *Candida* infections.

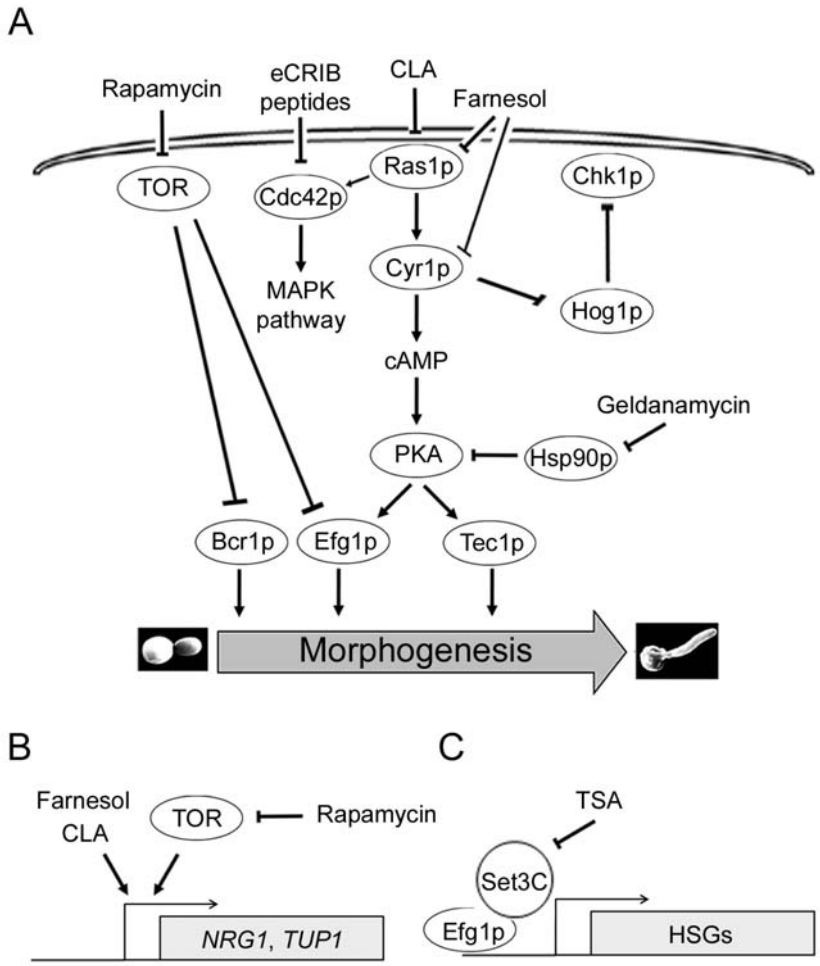

Figure 3. 1 Summary of the modes of action of selected small molecules which modulate morphogenesis in *Candida albicans*.

(A) Several small molecules, including farnesol (FOH), conjugated linoleic acid (CLA), rapamycin (Rapa), geldanamycin (GdA), mucin, diclofenac sodium, 1,4-diamino-2-butanone (DAB), hydroxyurea (HU), propranolol, and bathophenanthrolene disulfonic acid (BPS), modulate morphogenesis by targeting the Ras1p-cAMP-PKA signaling pathway and its downstream effectors. Notably, both CLA and GdA modulate morphogenesis independently of Efg1p. Extended CRIB (eCRIB) peptides interrupt the binding of the GTPase Cdc42p to its effectors Cst20p and Cla4p**,** and thus prevent the activation of the MAP kinase (MAPK) pathway. (B) Farnesol and CLA block the downregulation of *TUP1* and *NRG1* expression. In contrast, rapamycin induces the downregulation of both repressors by inhibiting TOR. (C) Trichostatin A (TSA) induces filamentation by relieving the Set3C-mediated repression of Efg1p-dependent transcription. TSA may also reduce *EFG1* transcript levels (see text for details). HSGs, hypha-specific genes.

Table V Small molecules that modulate morphogenesis in *C. albicans* by affecting the Ras1p-cAMP-PKA signaling pathway

Molecule	**Description/Target**	**Effects on *C. albicans***	**References**
farnesol	*Candida albicans*-produced QS molecule; downregulates Ras1p-cAMP-PKA pathway; increases *TUP1* mRNA levels	inhibits Y-H transition	(Davis-Hanna *et al.*, 2008; Hornby *et al.*, 2001; Kebaara *et al.*, 2008; Martins *et al.*, 2007; Sato *et al.*, 2004)
dodecanol	downregulates Ras1p-cAMP-PKA pathway	inhibits Y-H transition	(Davis-Hanna *et al.*, 2008; Hogan *et al.*, 2004; Martins *et al.*, 2007)
farnesoic acid	represses *EFG1*/*CPH1* expression levels in a *PHO81*-dependent manner	inhibits Y-H transition	(Chung *et al.*, 2005; Chung *et al.*, 2010; Oh *et al.*, 2001)
3-oxo-C12-acyl homoserine lactone (3OC12HSL)	*Pseudomonas aeruginosa*-produced QS molecule; targets Ras1p signaling	inhibits Y-H transition	(Hogan *et al.*, 2004)
cis-11-methyl-2-dodecenoic acid	*Xanthomonas campestris*-produced QS molecule; targets Ras1p signaling	inhibits Y-H transition	(Wang *et al.*, 2004)
cis-2-dodecenoic acid	*Burkholderia cenocepacia*-produced QS molecule; targets Ras1p signaling	inhibits Y-H transition	(Boon *et al.*, 2008)
unknown, found in supernatant	secreted by *Streptococcus gordonii*; affects activation of Cek1p, Mkc1p, Hog1p kinases	enhances hyphal development	(Bamford *et al.*, 2009)
conjugated linoleic acid	isolated from whey cream; affects Ras1p localization and reduces its cellular levels	inhibits Y-H transition	(Clement *et al.*, 2007)
diclofenac sodium	reduces *RAS1*/*EFG1* expression levels	inhibits Y-H transition; mildly affects growth rate	(Alem & Douglas, 2004; Ghalehnoo *et al.*, 2010)
salivary mucin	glycoprotein; downregulates *RAS1* expression levels	inhibits hyphal development	(Ogasawara *et al.*, 2007)

eCRIB peptides	impair the binding of the Rho GTPase Cdc42p to Cst20p and Cla4p	inhibit Y-H transition and hyphal elongation	(Su *et al.*, 2007)
trichostatin A	histone deacetylase inhibitor; reduces *EFG1* expression levels; relieves Set3C-mediated repression of Efg1-dependent transcription	inhibits Y-H transition in liquid medium; induces filamentation on solid medium	(Hnisz *et al.*, 2010; Simonetti *et al.*, 2007)
propranolol	calmodulin inhibitor; reduces *EFG1* mRNA levels	inhibits Y-H transition	(Baker *et al.*, 2002; Ueno *et al.*, 2009; Wolyniak & Sundstrom, 2007)
rapamycin	Tor1p inhibitor; affects Efg1p and Bcr1p; downregulates *TUP1* and *NRG1* expression levels	blocks filamentation on solid media ; induces cellular aggregation in Spider medium	(Bastidas *et al.*, 2009; Cutler *et al.*, 2001; Martins *et al.*, 2008)
geldanamycin	Hsp90p inhibitor; relieves Hsp90p-mediated repression of Ras1p-cAMP-PKA pathway	induces filamentation; toxic at high concentrations	(Shapiro *et al.*, 2009; Toenjes *et al.*, 2009)
bathophenanthrolene disulfonic acid	iron chelator; increases *EFG1* transcription	induces hyphal development	(Hameed *et al.*, 2008)
A-3	PKA inhibitor	inhibits Y-H transition	(Toenjes *et al.*, 2009)
1,4-diamino-2-butanone (DAB)	ornithine decarboxylase inhibitor; reduces *CYR1* expression	inhibits Y-H transition	(Ueno *et al.*, 2004)

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4. Chapter 4. Discussion and perspectives

4.1 Fatty acids inhibit hyphal growth in *Candida albicans*

An important component of *C. albicans*'s pathogenicity is its ability to switch from yeast growth to hyphal growth (Kumamoto & Vinces, 2005; Lo *et al.*, 1997; Saville *et al.*, 2003; Zheng & Wang, 2004). Lipidic compounds, such as farnesol and structurally related quorum sensing molecules have been reported to block the morphogenetic transition (Chapter 3). Similarly, various fatty acids, including butyric, capric, undecylenic, lauric, palmitoleic, conjugated linoleic, and arachidonic acids, have been shown to inhibit filamentation in *C. albicans* (Clement *et al.*, 2007; McLain *et al.*, 2000; Murzyn *et al.*, 2010; Noverr & Huffnagle, 2004; Shareck *et al.*, 2011). Conjugated linoleic acid (CLA), an 18-carbon long chain polyunsaturated fatty acid (PUFA), was chosen to characterize the effect of fatty acids on hyphal growth and explore their putative mode of action (Shareck *et al.*, 2011). Other PUFAs, such as oleic, linoleic, α- and γ-linolenic acids, exhibited hypha-inhibiting properties in *C. albicans* (Figure 4.1), in *Aspergillus fumigatus* (Clement *et al.*, 2008), and in *Fusarium graminearum* (data not shown). Additionally, CLA also abrogated filamentous growth in *C. tropicalis* and in *C. dubliniensis* (Figure 4.2). These results suggest that the response to fatty acids may be conserved among various *Candida* species and other fungi. Accordingly, farnesol blocked filamentation in *C. dubliniensis* (Martins *et al.*, 2007), inhibited growth and induced apoptosis in *Aspergillus nidulans* (Semighini *et al.*, 2006), inhibited conidiation in *Aspergillus niger* (Lorek *et al.*, 2008), and altered spore germination in *Fusarium graminearum* (Semighini *et al.*, 2008).

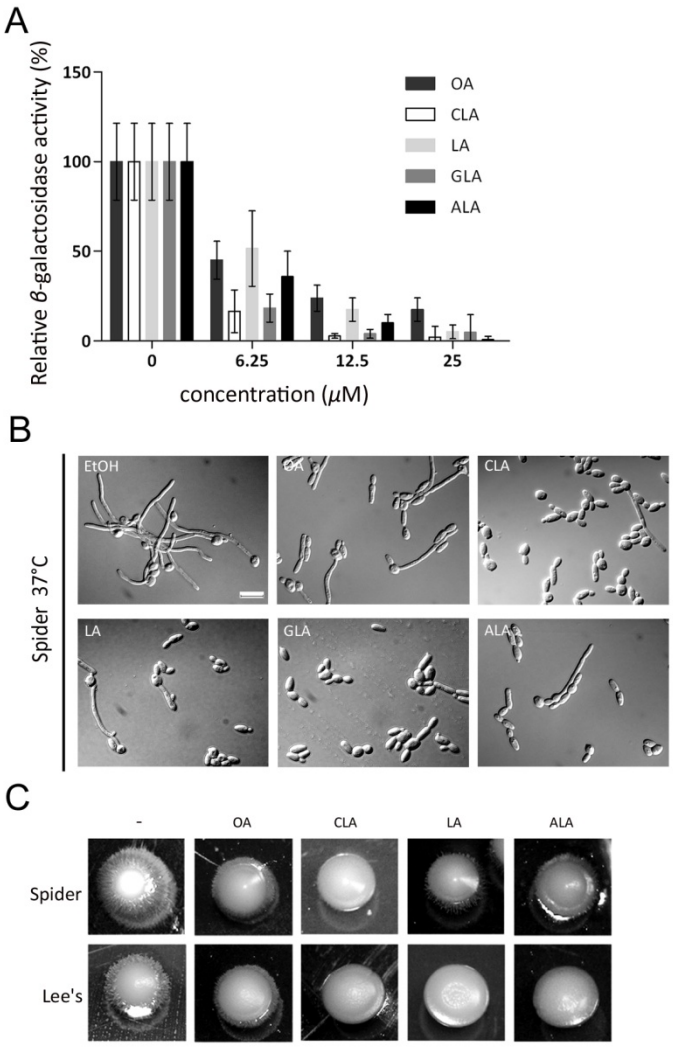

Figure 4. 1 Unsaturated fatty acids inhibit hyphal growth in *Candida albicans*.

(A) The effect of polyunsaturated fatty acids (PUFAs) on *C. albicans* hyphal growth was measured by using the *C. albicans* reporter strain *HWP1*p-lacZ. Cells were grown in Spider medium in absence or presence of PUFAs for 4 h at 37°C. Filamentation in PUFA-treated cultures was assessed by quantifying β-galactosidase activity, which was normalized to that of ethanol-supplemented cultures. Data are means and standard deviations of duplicate assays performed on three independent days. (B) Cells grown as in (A) were visualized by DIC microscopy at ×100 magnification. Bar = 10 μm. (C) *C. albicans* cells were grown on Spider and Lee's solid media supplemented with ethanol or PUFAs (100 μM) for 3-4 days at 37°C. oleic acid (OA); conjugated linoleic acid (CLA); linoleic acid (LA); α-linolenic acid (ALA); γ-linolenic acid (GLA).

Several fatty acids have been reported to exert cytotoxic properties towards *C. albicans* cells (Bergsson *et al.*, 2001; Carballeira, 2008; Deva *et al.*, 2000). Molecules that inhibit cellular growth are bound to impede hyphal growth, which questions whether fatty acids blocked filamentation merely by inhibiting overall growth. It was important to clearly demonstrate that while CLA interfered with hyphal growth, effective concentrations did not affect cellular growth. Toxicity and hypha-inhibiting properties of fatty acids in *C. albicans* depend on several factors, including concentration, growth medium, and inoculum. For instance, capric acid was toxic to *C. albicans* cells at 10 mM, causing disruption and disintegration of the plasma membrane, resulting in disorganization of the cytoplasm (Bergsson *et al*., 2001). In contrast, subinhibitory concentrations (10-300 μM) of capric acid inhibited hypha formation, but not cellular growth (Clement *et al.*, 2007; Murzyn *et al.*, 2010). Likewise, arachidonic acid inhibited growth of *C. albicans* cells at concentrations higher than 20 μM and blocked the yeast-to-hypha transition without affecting cell growth at 50 μM (Clement *et al.*, 2007; Deva *et al.*, 2000). In these experiments, different growth conditions and inocula accounted for the discrepancies. Similarly, several fatty acids used at 0.1 nM did not affect the yeast-to-hypha transition (Noverr & Huffnagle, 2004), while the same molecules interfered with hyphal growth at 100 μM (data not shown). Additionally, fatty acid susceptibility assays showed that the MIC_{50} of capric acid was medium-dependent (Clement *et al*., 2008). Taken together, these results indicate that growth conditions, fatty acid concentrations, and inocula dictate whether fatty acids affect general growth or hyphal development in *C. albicans*.

CLA did not block morphogenesis by inhibiting growth, which is why its mode of action was addressed. In addition, it was chosen to probe the mechanism by which fatty acids interfered with filamentation, given that it was the most effective of the molecules assayed (Figure 4.1A). However, it can only be assumed that CLA and other structurally similar PUFAs inhibit hyphal growth through the same mechanism.

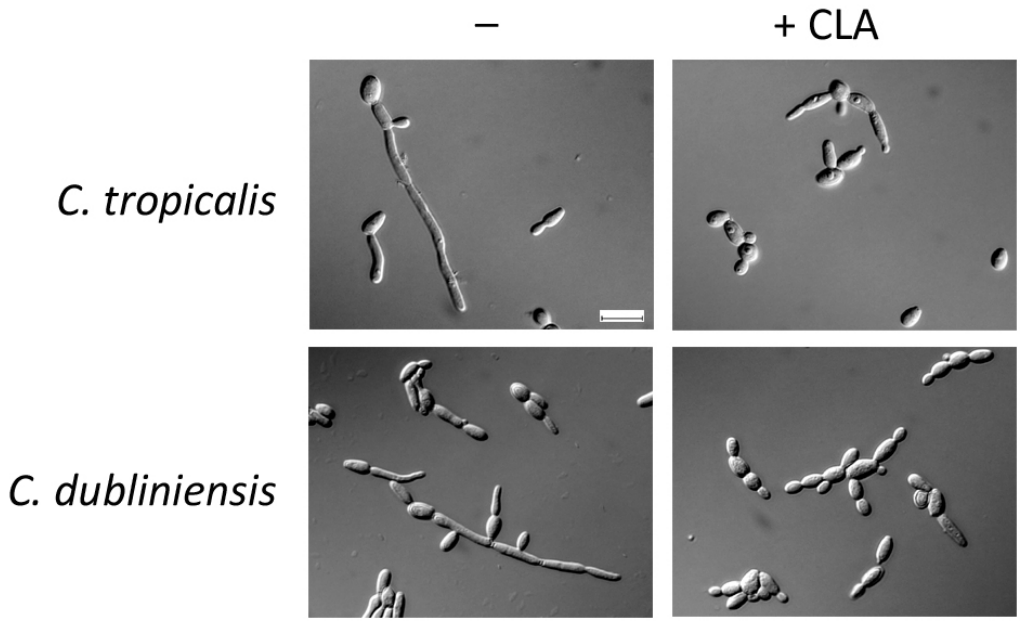

Figure 4. 2 Other *Candida* species respond to CLA.

CLA blocked filamentous growth in *C. tropicalis* and *C. dubliniensis*, suggesting that CLA modulates filamentous growth in other *Candida* species. Cells were visualized by DIC microscopy at ×100 magnification. Bar = 10 μm.

4.2 Using transcriptional profiling to address the mode of action of CLA

CLA, as well as other fatty acids, were deemed to be bioactive molecules on the basis that they inhibited hyphal growth in *C. albicans*. In contrast to molecules that target specific gene products or signaling pathways, e.g. rapamycin, geldanamycin, and histone deacetylase inhibitors (reviewed in Chapter 3), CLA does not have a known cellular target, even less so a target mediating its effect on filamentation. In this respect, CLA is similar to farnesol: receptors or binding proteins for farnesol still remain to be identified, although the GTPase Ras1p and/or the adenylate cyclase Cyr1p may be potential targets (Davis-Hanna *et al.*, 2008). Thus, gene expression profiling was conducted to address the mechanism by

which CLA inhibited the yeast-to-hypha transition in *C. albicans*. This approach was chosen to gain insight into the transcriptional response of cells to CLA on a genome-wide scale. Although not novel, this type of experimental approach has been proposed as a means to elucidate the mechanisms of small molecules (Butcher & Schreiber, 2005). Additionally, transcriptional profiling has been used to examine the response of *C. albicans* to farnesol (Cao *et al.*, 2005; Cho *et al.*, 2007; Enjalbert & Whiteway, 2005; Uppuluri *et al.*, 2007) and to rapamycin (Bastidas *et al.*, 2009), which are two molecules that modulate morphogenesis.

Gene expression analysis generates a wealth of data, from which genes or signaling pathways involved in a specific phenotype/response can be identified, thus constituting a starting point to direct further studies. The transcriptional profiles of cells undergoing the yeast-to-hypha transition treated or not with CLA were obtained independently by using a common control condition, and were then compared to one another. The microarray experiments were designed as such in order to (i) characterize independently the transcriptional program regulating hyphal growth in Spider medium and the transcriptional changes elicited by CLA and (ii) identify the genes or signaling pathways potentially mediating CLA's hypha-inhibiting effects.

4.2.1 Hyphal growth program in Spider medium

The yeast-to-hypha transition is induced in cells transferred from YPD at 30°C to Spider at 37°C (Figure 2.2A). The transcriptional profile of cells undergoing the yeast-to-hypha transition in Spider medium revealed that 520 genes were modulated upon growth at 37°C (Table S1). Of those genes, 198 were upregulated while 322 were downregulated. Most of the downregulated transcripts encoded gene products involved in RNA metabolic processes, ribosome biogenesis, translation, and transcription, which reflects a repression of the translational machinery. Accordingly, genes involved in protein translation were also downregulated in cells undergoing the yeast-to-hypha transition in serum-containing YPD

and Lee's media (Nantel *et al.*, 2002). Among the subset of upregulated genes, ~30% of the transcripts have been described as being induced during the yeast-to-hypha transition in Lee's medium (Goyard *et al.*, 2008) (Table S1). Spider and Lee's are hypha-inducing media that contain a source of fermentable carbon, various amino acids, and salts, which may explain the similarities between both hyphal growth programs.

Transcriptional profiling of the yeast-to-hypha transition did not reveal the existence of a novel gene product or signaling pathway regulating specifically the Spider-induced hyphal growth program. Instead, several signaling pathways appeared to regulate filamentation concomitantly. Expression levels of key regulators of hyphal growth including *CPH1*, *CPH2*, *TEC1*, *BCR1*, *UME6*, *GAT2*, *RAS1*, *CYR1*, *CAS4*, and *MSB2* were upregulated (Table IV). Except for *BCR1*, the products of these genes are required for filamentation in different hypha-inducing conditions (Banerjee *et al.*, 2008; Homann *et al.*, 2009; Lane *et al.*, 2001a; Lane *et al.*, 2001b; Leberer *et al.*, 2001; Liu *et al.*, 1994; Rocha *et al.*, 2001; Roman *et al.*, 2009b; Schweizer *et al.*, 2000; Song *et al.*, 2008; Zeidler *et al.*, 2009). The transcriptional data suggest that each of these regulators contributes to hyphal growth in Spider medium. However, this was not the case, as strains deleted for *CPH1*, *CPH2*, and *UME6* still underwent the yeast-to-hypha transition in Spider medium (Figure 4.3). Although not verified, *MSB2* may also be dispensable for filamentation, as a *msb2/msb2* mutant strain filamented in serum-containing medium (Roman *et al*., 2009b). Among the candidate genes involved in Spider-induced filamentation, *RAS1* and *TEC1* were necessary for hyphal growth (data not shown), while *CYR1*, *CAS4*, and *GAT2* may also be required for filamentation (Fang & Wang, 2006; Homann *et al.*, 2009; Noble *et al.*, 2010; Rocha *et al.*, 2001; Song *et al.*, 2008).

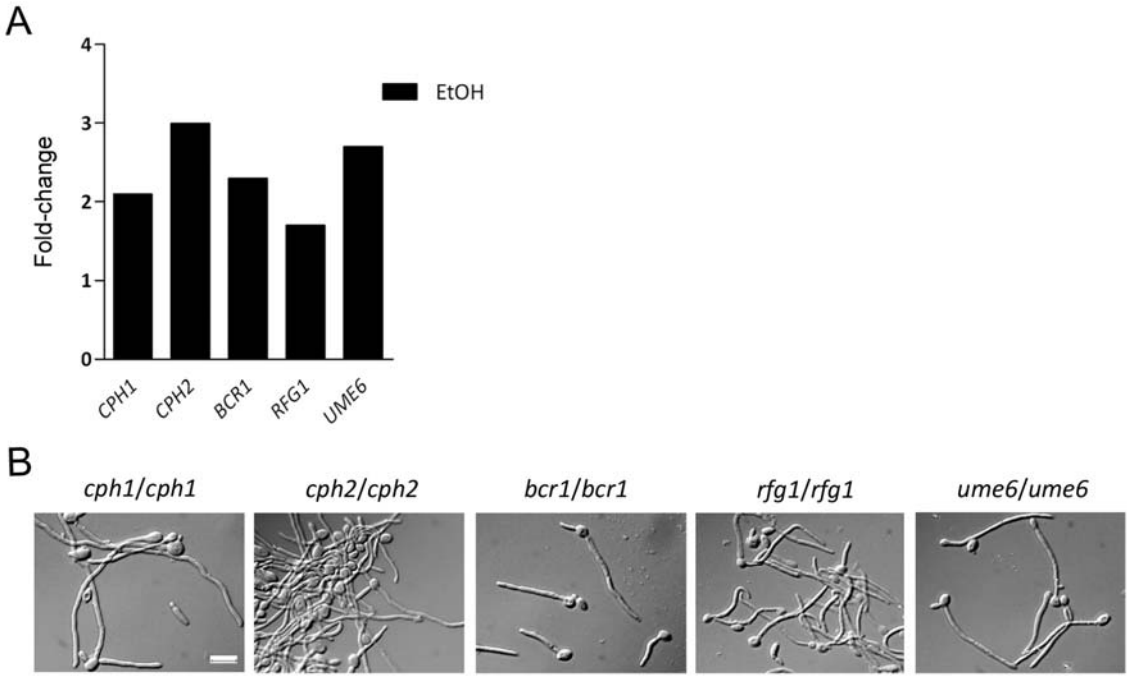

Figure 4. 3 Differentially expressed transcription factors dispensable for hyphal growth.

(A) Fold-change of genes encoding transcription factors in cells undergoing the yeast-to-hypha transition in Spider medium at 37°C. The significantly differentially expressed genes were obtained by comparing growth in Spider medium at 37°C to that at 30°C (Table S1). Data are fold-change values. (B) *cph1/cph1* (JKC19), *cph2/cph2* (HLY1921), *bcr1/bcr1* (CJN702), *rfg1/rfg1* (DK129) and *ume6/ume6* (DK312) strains were grown in Spider medium at 37°C for 4 h. Cells were visualized using DIC optics at ×100 magnification. Bar = 10 μm.

How does one explain that genes encoding transcription factors induced during the yeast-to-hypha transition are dispensable for filamentation? Before presenting the concept of secondary effect genes, one has to bear in mind that hyphal growth is regulated by a complex network of signaling pathways, which lie parallel to or converge onto one another (Figure 1.8). In Spider medium, the yeast-to-hypha transition is controlled mainly by the Ras1p-cAMP-PKA signaling pathway, while other pathways, such as the MAP kinase and Cph2p-dependent pathways, play minor roles. *CPH1* and *CPH2* transcript levels are induced as a result of hyphal growth being driven by the Ras1p signaling pathway. Their

modulation is merely a consequence, a secondary effect of an induced hyphal growth program. Hence, secondary effect genes are genes that are differentially expressed, but that are not required for a specific phenotype, e.g. *CPH1* and *CHP2* in Spider-induced filamentation. The induction of *UME6* also stems from a secondary effect, given that it is a downstream target of most signaling pathways and is induced by upstream regulators such as Ras1p and Tec1p (Zeidler *et al*., 2009). Additionally, the upregulation of Tup1-regulated *GAT2* may be the result of Ras1p-dependent induction of hyphal growth combined to Tup1p-dependent derepression of filamentation (Garcia-Sanchez *et al.*, 2005; Kadosh & Johnson, 2005). Thus, when analyzing transcriptional data, one has to beware of secondary effect genes. The combination of transcriptional profiling with other methods, i.e. phenotypic screening of deletion strains, enables one to eliminate such genes, an important step when filtering transcriptional data (discussed in 4.2.3).

4.2.2 Genes upregulated by CLA

Transcriptional profiling revealed that hallmark genes of β-oxidation, the glyoxylate cycle, and gluconeogenesis were upregulated in CLA-treated cells, suggesting that this fatty acid could be converted to glucose (Table S2-S4). *C. albicans* has the ability to use alternative carbon sources, including fatty acids such as myristic, palmitic, oleic, linoleic, linolenic, and arachidonic acids as sole carbon sources (Deva *et al.*, 2000; Ramirez & Lorenz, 2007; Ramirez & Lorenz, 2009). Fatty acid metabolism involves β-oxidation, the glyoxylate cycle, and gluconeogenesis enzymes, which convert fatty acids to acetyl-CoA and then to sugar phosphates, while replenishing tricarboxylic acid (TCA) cycle intermediates to generate energy.

Upon the yeast-to-hypha transition, a metabolic shift from aerobic respiration to fermentation occurs. Indeed, genes involved in glycolysis and fermentation were shown to be upregulated during filamentation, most of them being Efg1p-regulated (Bahn *et al.*, 2007; Doedt *et al.*, 2004). This shift is required for hyphal growth, as glycolytic inhibitors

interfered with the yeast-to-hypha transition in *C. albicans* (Land *et al.*, 1975a). It was ruled out that fatty acid metabolism interfered with metabolic specialization of cells induced to filament by confirming that CLA inhibited hyphal growth in mutant strains which are unable to metabolize fatty acids (Ramirez & Lorenz, 2007; Ramirez & Lorenz, 2009) (Figure 4.4). The upregulation of genes involved in fatty acid metabolism was a secondary effect of the response of cells to CLA. In light of these findings, similar microarray experiments using strains defective in fatty acid metabolism could be performed to reduce the amount of transcriptional data, yielding cleaner transcriptional profiles, devoid of differentially expressed secondary effect genes.

Fatty acids are not only used as nonfermentable carbon sources, but they are also derived into oxygenated lipid metabolites (oxylipins) such as eicosanoids, which include prostaglandins and leukotrienes (Erb-Downward & Huffnagle, 2006). While eicosanoids are typically derived from arachidonic acid, 18-carbon PUFAs such as linoleic and α-linolenic acids can also serve as precursors for the synthesis of oxylipins (Noverr *et al.*, 2003). This notion is relevant because eicosanoids PGE2 and TXB2 have been reported to promote filamentation in *C. albicans* (Kalo-Klein & Witkin, 1990; Noverr & Huffnagle, 2004). *OLE2* and *FET3* appear to be involved in prostaglandin synthesis (Erb-Downward & Noverr, 2007). Thus, it cannot be excluded that CLA is converted into oxylipins which, in turn, are responsible for its hypha-inhibiting properties. Examining CLA's effect in strains in which prostaglandin synthesis is defective, i.e. deleted for *OLE2* and/or *FET3*, could shed light on the role of prostaglandins in the CLA-mediated hyphal growth inhibition.

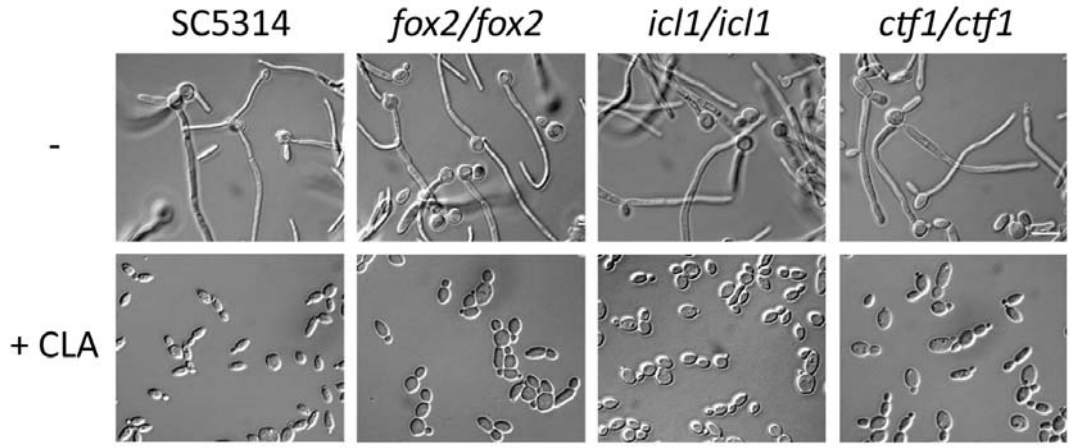

Figure 4. 4 Inhibition of hyphal growth by CLA does not depend on its metabolism.

Parental (SC5314) and mutant strains *fox2/fox2* (MRC6), *icl1/icl1* (MRC10) and *ctf1/ctf1* (MRC41) strains were grown in Spider medium at 37°C in the absence or presence of 100 μM CLA for 4 h. Cells were visualized using DIC optics at ×100 magnification. Bar = 10 μm.

4.2.3 Genes downregulated by CLA

CLA reduced the magnitude of expression of hypha-specific genes (HSGs) and genes encoding morphogenesis regulators (Table S4). Expression levels of hyphal markers, such as *HWP1*, *ALS3*, and *RBT1*, were greatly reduced in CLA-treated cells compared to untreated cells (Table S4). However, except for *HGC1*, HSGs are not essential for morphogenesis, suggesting that they do not mediate CLA's effect on the yeast-to-hypha transition (Zheng & Wang, 2004). CLA modulated the expression of several genes encoding transcription factors and regulators of hyphal growth (Figure 2.3C). Because they are involved in morphogenesis, *GAT2*, *UME6*, *TEC1*, *RFG1 RAS1*, *BEM2*, and *MSB2* appeared to be potential targets of CLA. Screening strains deleted for several of these genes revealed that *UME6* and *RFG1* were not required for the CLA-mediated hyphal growth inhibition (Figure 2.4). Thus, *UME6* and *RFG1* are modulated as a result of CLA affecting

another target and are thus secondary effect genes. The examination of deletion strains revealed that *RAS1* and *TEC1* were both required for hyphal growth in Spider medium (data not shown). Given that *RAS1* and *TEC1* function in the same pathway, these findings suggest that CLA may inhibit filamentation by affecting the Ras1p signaling pathway. This assumption was based on a phenotypic screen that was by no means exclusive. Since strains deleted for *GAT2*, *MSB2*, and *BEM2* were not screened, it cannot be excluded that one of these genes encodes the target of CLA which mediates the fatty acid's effect on hyphal growth.

The mode of action of a small molecule can be investigated using gene expression profiling, which generates the global transcriptional effects exerted by a molecule in a given context (specific process, growth conditions, etc.). In the present study, the transcriptional effects of CLA on the yeast-to-hypha transition in *C. albicans* were examined. Given that such an approach generates extensive data, extrapolating a mechanism of action might turn out to be a laborious task. First, the target(s) of a small molecule may not be modulated at the transcriptional level. Second, the genes that are bound to be modulated tend to be downstream effectors which are not necessarily involved in a given effect. For instance, genes involved in fatty acid metabolism and hypha-specific genes are the most differentially expressed genes in response to CLA, but none play a role in the CLA-mediated hyphal growth inhibition. Third, not all genes have a known biological function.

Because of these disadvantages, it is essential to use other methods in conjunction with transcriptional profiling in order to identify cellular targets mediating a phenotype. Screening collections of *C. albicans* mutant strains for their capacity to filament in Spider medium and for their response to CLA could constitute a means to identify potential CLA targets, i.e. genes encoding activators of Spider-induced filamentation or repressors mediating CLA's effect on hyphal growth. Two sets of *C. albicans* deletion strains are available, one of 143 strains deleted for transcriptional regulators and another of 674

homozygous deletion strains (Homann *et al.*, 2009; Noble *et al.*, 2010). The screening of these collections has shown that *GAT2*, orf19.4998, and *TEC1* were essential for filamentation induced on solid Spider medium. Since these genes regulate filamentation in Spider medium and they were CLA-modulated (Table S4), they may constitute CLA targets, as their respective deletions phenocopy CLA's effect on hyphal growth. In fact, *TEC1* is an indirect target of CLA because it lies downstream of Ras1p, which is the primary target of CLA (Figure 2.5; discussed in 4.3). By combining a phenotypic screen with gene expression analysis, transcriptionally-modulated targets can be validated, secondary effect genes can be eliminated, and genes that are not modulated transcriptionally or that have not been annotated can be identified.

4.3 CLA targets Ras1p signaling

4.3.1 CLA affects GFP-Ras1p levels and its subcellular localization

RAS1 encodes one of the two Ras GTPases of *C. albicans*. Unlike its *S. cerevisiae* homologue, *RAS1* is not essential for cell viability. However, it is required for hyphal growth under a variety of hypha-inducing conditions, including Spider medium (Leberer *et al.*, 2001; Zhu *et al.*, 2009). *RAS1* mRNA and protein levels increased at the onset of the yeast-to-hypha transition (Figure 2.6A and B). CLA prevented *RAS1* induction. Instead, transcript levels remained comparable to levels at the zero time point (Figure 2.6A), suggesting that CLA may interfere directly or indirectly with an activator of *RAS1* transcription (discussed in 4.5). Thus, based on its mRNA levels, Ras1p cellular levels should have remained unchanged compared to levels at the zero time point. Unexpectedly, GFP-Ras1p protein levels decreased in CLA-treated cells (Figure 2.6B).

Why do GFP-Ras1p protein levels decrease in presence of CLA? Reduced protein levels can be due to a decrease in mRNA transcription or translation or to mRNA or protein

degradation. In presence of CLA, *RAS1* transcript levels remained constant, indicating that a decrease in transcription or mRNA degradation did not account for reduced GFP-Ras1p protein levels. Additionally, transcriptional profiling revealed that 61 genes encoding components of the translational machinery were repressed at 37°C, whether CLA was present or not (Figure S1B). Thus, it is unlikely that reduced GFP-Ras1p levels were caused by a halt in translation, as this decrease would not have been specific to CLA-treated cells. In fact, the downregulation of translation has been observed in *C. albicans* cells grown in presence of serum or phagocytosed (Lorenz *et al.*, 2004; Nantel *et al.*, 2002).

Protein degradation may account for the decline in GFP-Ras1p levels. Abnormal, short-lived, damaged, denatured, or delocalized proteins undergo degradation (Ciechanover *et al.*, 2000; Paiva *et al.*, 2009; Roman *et al.*, 2009a). Strikingly, GFP-Ras1p appeared delocalized from the plasma membrane in CLA-treated cells (at 30°C) (Figure 2.6C; 4.6). In these conditions, i.e. Spider 30°C, CLA also reduced *RAS1* mRNA and protein levels (Figure 4.5). Several lines of evidence indicate that the delocalization of Ras from the plasma membrane influences its cellular levels. For instance, in *S. cerevisiae*, a Ras2p mutant that was not palmitoylated was delocalized from the membrane and had lower cellular levels compared to the wild-type protein (Deschenes & Broach, 1987). In EJ cells, farnesylthiosalicylic acid dislodged Ras from its membrane anchorage sites, reducing membrane-bound Ras levels, accelerating its degradation, and decreasing its cellular levels (Haklai *et al.*, 1998). It is tempting to speculate that in CLA-treated cells, the delocalization of GFP-Ras1p from the plasma membrane results in its degradation, thus reducing its protein levels (Figure 2.6).

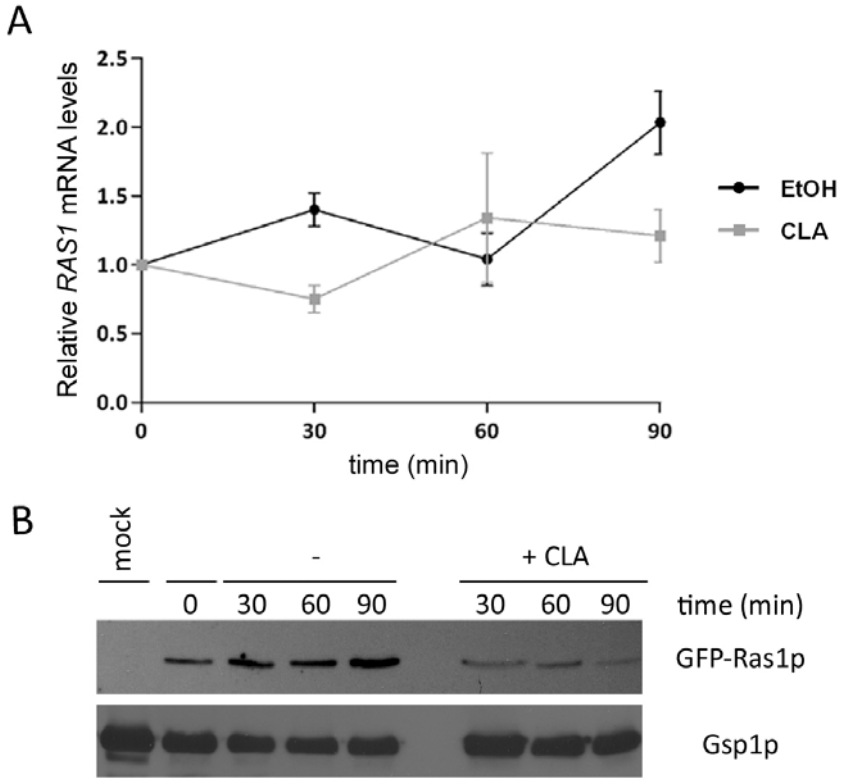

Figure 4. 5 CLA decreases *RAS1* mRNA and protein levels.

(A) SC5314 cells were grown in Spider medium at 30°C in the absence or presence of 25 μM CLA and harvested at the indicated time points. Transcript levels of *RAS1* were measured by quantitative PCR and normalized to those for *ACT1*. Relative expression levels were obtained by normalizing data for each time point to data obtained at time zero. Data are means and standard deviations for duplicate biological samples. (B) Ras1p protein levels were analyzed using a strain expressing GFP-Ras1p. Total protein extracts were prepared from SC5314 (mock) and GFP-Ras1p (WY-ZXD3) strains grown as described for panel A in the absence or presence of 100 μM CLA. Western blotting analysis was performed using anti-GFP antibodies. Gsp1p, shown as a loading control, was detected using antibodies raised against *S. cerevisiae* Gsp1p.

Alternatively, the degradation of GFP-Ras1p may be an artefact stemming from the nature of the protein, in that GFP-Ras1p is a fusion protein. Delocalized from the plasma membrane, GFP-Ras1p may be recognized as an abnormal protein and subsequently degraded. However, GFP fusion proteins have been used as tools to study protein localization, expression, and turnover. In *S. cerevisiae*, a GFP fusion protein was used to study the protein turnover of the lactate permease Jen1p in response to glucose (Paiva *et al.*, 2002). In *C. albicans*, a GFP-Ras1p-expressing strain was used to examine the subcellular localization and kinetics of expression of Ras1p (Zhu *et al*., 2009). Although it is unlikely that reduced GFP-Ras1p levels were caused by the degradation of the fusion protein, these results should be validated by examining Ras1p endogenous levels using either specific home-made and tested or commercial antibodies. However, commercial antibodies that specifically recognize the *C. albicans* Ras1p protein are currently unavailable.

To further substantiate the findings pertaining to the delocalization of GFP-Ras1p from the plasma membrane and its potential degradation, several experiments could be performed. First, cell fractionation could provide a means to confirm the delocalization of GFP-Ras1p as demonstrated by microscopic observation and to assess quantitatively the abundance of GFP-Ras1p in membranes and in the cytosol. Second, it could be interesting to examine how GFP-Ras1p is degraded, either via the proteasome or the vacuole/lysosome. The *pre1* conditional mutant strain, in which the proteasome machinery can be impaired, could be used to determine whether or not the proteasome is involved in Ras1p degradation (Roman *et al.*, 2009a). Staining of vacuoles and colocalization studies could be used to determine whether or not Ras1p is targeted to the vacuole for degradation (Kruckeberg *et al.*, 1999; Paiva *et al.*, 2002).

4.3.2 Causes of GFP-Ras1p delocalization

Ras proteins undergo posttranslational modifications by lipids. Such modifications are important for Ras membrane targeting, subcellular localization, and effector

interactions. Ras proteins terminate with a CAAX tetrapeptide sequence which, once lipid-modified, enables membrane anchorage (Wennerberg *et al.*, 2005). Prenylation is the addition of a farnesyl group to the cysteine residue of the CAAX motif catalyzed by farnesyl transferase, while palmitoylation consists in the addition of the fatty acid palmitate to a cysteine residue near the carboxyl end (Figure 1.14). Prenylation is required for the association of Ras to ER and Golgi endomembranes, while palmitoylation allows Ras to be anchored stably within the plasma membrane (Hancock, 2003; Linder & Deschenes, 2007). Interestingly, when Ras prenylation was blocked using a protein farnesyl transferase inhibitor, hyphal growth was blocked in *C. albicans* (McGeady *et al.*, 2002). These findings suggest that Ras posttranslational modifications play a role in the yeast-to-hypha transition in *C. albicans*.

CLA treatment results in the delocalization of GFP-Ras1p and in the inhibition of hyphal growth. Both of these effects may be due to the fatty acid interfering with the posttranslational modifications of Ras1p. Prenylation involves two enzymes: HMG-CoA reductase, which produces the farnesylpyrophosphate precursor mevalonate, and farnesyl transferase I, which catalyzes the attachment of the farnesyl group (Collett *et al.*, 2001; Nickerson *et al.*, 2006). CLA's effect on prenylation could be determined by quantifying HMG-CoA reductase transcript and/or protein levels and/or by assessing farnesyl transferase activity, measured by the incorporation of radiolabeled farnesylpyrophosphate (Collett *et al*., 2001). Interestingly, there is evidence that farnesol promotes the posttranscriptional degradation of HMG-CoA reductase in mammalian cells (Correll *et al.*, 1994; Meigs & Simoni, 1997). It is not known whether or not the same phenomenon occurs in farnesol-treated *C. albicans* cells. However, according to transcriptional data, *HMG1* expression levels were not differentially expressed, suggesting that CLA does not affect HMG-CoA reductase mRNA levels (Table S7). CLA's effect on palmitoylation could be determined by measuring the incorporation of radiolabeled palmitate which reflects the activity of palmitoyl transferase (Collett *et al.*, 2001; Deschenes & Broach, 1987). While it cannot be excluded that CLA affects GFP-Ras1p's localization by interfering with its

posttranslational modifications, such a hypothesis appears rather unlikely, given that in mammalian cells, long-chain PUFAs modulated Ras localization without affecting its posttranslational processing (Collett *et al.*, 2001; Davidson *et al.*, 1999).

Alternatively, the delocalization of GFP-Ras1p may be an indirect effect of CLA affecting another cellular component, e.g. the plasma membrane, which constitutes one of the microenvironments of Ras. The lipidated C-terminus of Ras anchors it to the inner leaflet of the plasma membrane (Brunsveld *et al.*, 2009). As a peripheral protein, Ras does not transverse the plasma membrane, but is rather only associated to it. Peripheral proteins are subject to changes in their lipidic microenvironment, which can influence their localization (van der Rest *et al.*, 1995). For instance, the PUFA docosahexaenoic acid (DHA) reduced the abundance of membrane-bound Ras in mouse colon cells by altering the plasma membrane structure (Collett *et al.*, 2001; Seo *et al.*, 2006). Indeed, DHA-enriched membranes were suggested to have a looser packing, thereby weakening van der Waals interactions between anchors of lipidated proteins and phospholipid acyl chains (Seo *et al*., 2006). Based on this line of evidence, it is possible that CLA indirectly affects GFP-Ras1p localization by altering the plasma membrane structure (discussed in 4.3.3; 4.3.4).

4.3.3 A link between plasma membrane properties and hyphal growth

n-3 PUFAs as well as CLA have been suggested to alter basic properties of cell membranes, including phospholipid acyl chain order, fluidity, lipid rafts, and microdomain organization (Chapkin *et al.*, 2008; Ma *et al.*, 2004; Stillwell & Wassall, 2003). In *C. albicans*, several membrane properties appear to influence hyphal development. For instance, the balance between unsaturated and saturated fatty acids is critical for hyphal growth, but membrane fluidity is not (Krishnamurthy *et al.*, 2004; Xu *et al.*, 2009). In addition, lipid rafts, which are membrane microdomains enriched in sterols and sphingolipids, play a role in filamentation (Martin & Konopka, 2004). Thus, the CLA-

mediated hyphal growth inhibition may be due to the fatty acid modulating a property of the plasma membrane.

RTA3, which encodes an integral membrane transporter, was induced in CLA-treated cells (Table S4). This result was initially taken as genetic evidence that the fatty acid was affecting the membrane. Indeed, *RTA3* is homologous to the *S. cerevisiae* flippase *RSB1*, whose expression is induced when plasma membrane glycerophospholipid asymmetry is altered (Ikeda *et al.*, 2008). In an attempt to further demonstrate that CLA modulated the plasma membrane, sensitivity of untreated and CLA-treated cells to various membrane perturbing agents, including hygromycin B, calcofluor white, and lithium chloride, was examined. However, CLA did not alter sensitivity of cells to the aforementioned agents, suggesting that CLA's effect on the membrane may have been too mild to detect using this type of assay (data not shown). Alternatively, it may be more relevant to determine whether or not CLA modulates cellular ratios of unsaturated to saturated fatty acids and membrane unsaturation levels and/or interferes with the polarization of lipid rafts, as such properties are known to impact hyphal growth in *C. albicans* (Krishnamurthy *et al.*, 2004; Martin & Konopka, 2004).

4.3.4 Potential mechanism for the CLA-mediated delocalization of GFP-Ras1p

Microscopic observations showed that in *C. albicans*, GFP-Ras1p was already membrane-bound prior to hyphal induction (Figure 4.6, upper panel). In untreated cells, levels of GFP-Ras1p at the membrane were maintained throughout time, as reflected by the consistent fluorescent rim. In contrast, 30 minutes upon CLA treatment, levels of membrane GFP-Ras1p started to decrease (Figure 4.6), and remained low at later time points (Figure 2.6C; data not shown). Additionally, intracellular fluorescent clusters appeared, suggesting a localized accumulation of GFP-Ras1p. How may one explain CLA's effect on GFP-Ras1p localization, i.e. its reduced levels at the plasma membrane and its relocalization to intracellular clusters? Does CLA dislodge GFP-Ras1p from the

membrane or does it prevent newly synthesized GFP-Ras1p from being properly anchored in the membrane? How can evidence pertaining to the subcellular localization and trafficking of Ras proteins accommodate these findings?

Ras undergoes depalmitoylation/palmitoylation cycles, enabling it to traffic to and from the plasma membrane. This continuous cycle occurs on a time scale of minutes and maintains the steady-state distribution between Ras membrane and intracellular pools (Rocks *et al.*, 2005). Thus, in both untreated and CLA-treated cells, the membrane-bound pool of GFP-Ras1p may be depalmitoylated at the membrane, relocalized to other membranes, repalmitoylated at the ER/Golgi complex, and retargeted to the plasma membrane (Figure 1.15). In untreated cells, Ras1p trafficking probably proceeds unperturbed, which would account for the steady-state levels of membrane-bound GFP-Ras1p (Figure 4.6). In contrast, CLA may prevent lipidated GFP-Ras1p from being properly anchored in the membrane. Indeed, there is evidence that unsaturated membranes do not constitute an environment conducive to the attachment of lipidated proteins such as Ras (Seo *et al*., 2006). Thus, repalmitoylated GFP-Ras1p may be delivered to the plasma membrane via the classical secretory pathway, but may be unable to associate with it, resulting in decreased levels of membrane-bound GFP-Ras1p.

This model remains purely speculative, as it is still unclear whether or not the Ras depalmitoylation/palmitoylation cycle operates in yeast (Linder & Deschenes, 2007). In *S. cerevisiae*, Ras trafficking from the ER to the plasma membrane does not seem to involve the secretory pathway (Linder & Deschenes, 2007; Wang & Deschenes, 2006), although in *Cryptococcus neoformans*, Ras proteins appeared to sort through the Golgi (Nichols *et al.*, 2009; Omerovic *et al.*, 2007). Interestingly, the intracellular clusters in which Ras proteins accumulated resembled the fluorescent clusters visible in CLA-treated cells (Figure 4.6). Staining CLA-treated cells with a vesicle dye could help confirm the nature of these structures. Additionally, in *S. cerevisiae*, functional mitochondria appeared to be involved in the trafficking of Ras to the plasma membrane (Wang & Deschenes, 2006). Strikingly,

CLA had a strong effect on the expression of mitochondrial genes (data not shown), suggesting an association between the fatty acid, mitochondria, and Ras trafficking to the membrane. These results warrant the further examination of mitochondria, which could be labeled with MitoTracker, a mitochondrion-specific dye, to perform colocalization studies with GFP-Ras1p.

Another factor that needs to be addressed is the time frame. Indeed, the proposed model depends on CLA altering the plasma membrane structure by increasing its unsaturation levels. To do so, CLA first has to be incorporated into membrane phospholipids. If unsaturated, CLA-enriched membranes affect the localization of GFP-Ras1p as early as 30 minutes upon treatment with the fatty acid, then CLA must be incorporated into membrane phospholipids within a similar time frame. Such an issue has not been addressed *per se* in *C. albicans*. However, in *S. cerevisiae*, there is evidence that oleic acid treatment changes the composition of membrane phospholipids within 30 minutes, increasing the cellular ratio of unsaturated to saturated fatty acids (Petschnigg *et al.*, 2009). Moreover, in *C. albicans* biofilm cells, a 48-h treatment with 1 mM of arachidonic acid increased the unsaturation index of membrane phospholipids (Ells *et al.*, 2009). Thus, CLA treatment may increase unsaturation levels of membrane phospholipids, thus creating an environment in which lipidated GFP-Ras1p cannot be anchored properly, resulting in its delocalization from the plasma membrane. Examining the fatty acyl composition of membrane lipids in CLA-treated cells could lend support to this hypothesis.

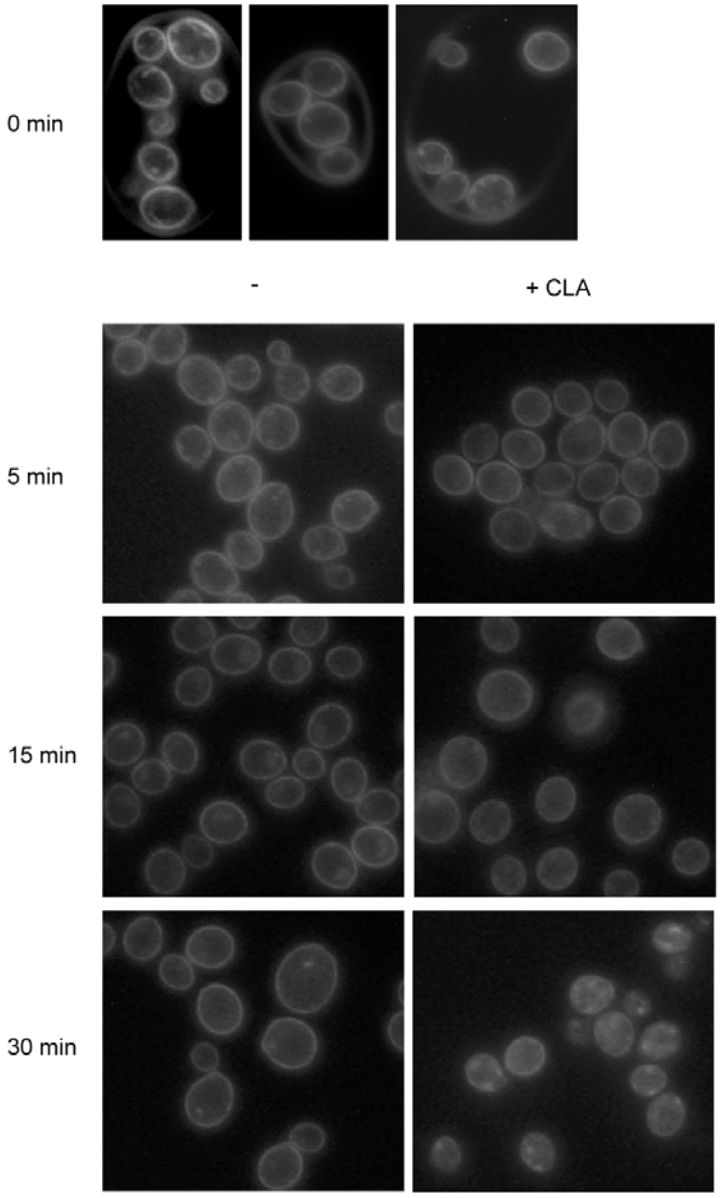

Figure 4. 6 Effect of CLA on the subcellular localization of GFP-Ras1p in *C. albicans*.

GFP-Ras1p-expressing cells were grown in Spider medium at 30°C in the absence or presence of 100 μM CLA. Aliquots of cells were removed and examined directly at ×100 magnification using epifluorescence.

4.3.5 Downstream consequences of CLA-modulated Ras1p signaling

4.3.5.1 Activation of the MAP kinase pathway

CLA impacted GFP-Ras1p in two ways: first, by reducing its cellular levels, and second, by causing its delocalization from the plasma membrane (Figure 2.6). Although Ras has been reported to signal from various cellular locations, in *S. cerevisiae*, the cytoplasmic Ras2p was 100-times less effective than the plasma membrane-localized Ras2p (Hancock, 2003; Kuroda *et al.*, 1993). Thus, suboptimal GFP-Ras1p localization and concentrations are bound to impede Ras1p signaling, including the activation of the MAP kinase and the cAMP-PKA signaling pathways (Leberer *et al*., 2001). The phosphorylation status of the ERK-like kinase Cek1p reflects the activity of Ras1p. In presence of CLA, Cek1p was not phosphorylated, indicating the fatty acid interfered with Ras1p signaling (Figure 4.7A). Likewise, the hypha-inhibiting molecule farnesol also repressed Cek1p phosphorylation (Roman *et al*., 2009a). However, CLA-mediated hyphal growth inhibition did not depend on *CEK1* (Figure 4.7B). The apparent inactivation of the MAP kinase pathway is not the cause, but rather the consequence of CLA inhibiting filamentation by affecting another signaling pathway, i.e. the cAMP-PKA pathway.

4.3.5.2 Activation of the cAMP-PKA-Efg1p pathway

Ras1p interacts with the Ras-association domain of the adenylate cyclase Cyr1p to promote cAMP synthesis (Fang & Wang, 2006). cAMP binds to the regulatory subunit of PKA (Bcy1p), causing a conformational change and releasing the catalytic subunits Tpk1p and Tpk2p, which are free to activate downstream effectors. Efg1p is the potential target of PKA, as it harbors a potential phosphorylation site for A-type kinases at T206 (Bockmuhl & Ernst, 2001; Sonneborn *et al.*, 2000). In hypha-inducing conditions, Efg1p is activated by PKA isoforms Tpk1p and Tpk2p. Activated Efg1p downregulates its own promoter activity, thereby reducing *EFG1* expression levels (Tebarth *et al*., 2003). The transcription factor *TEC1*, partially regulated by Efg1p, is expressed in response to hypha-inducing conditions. Given that the activation of the cAMP-PKA-Efg1p pathway depends on Ras1p,

CLA's effects on several of the aforementioned molecular events or components of the pathway were examined.

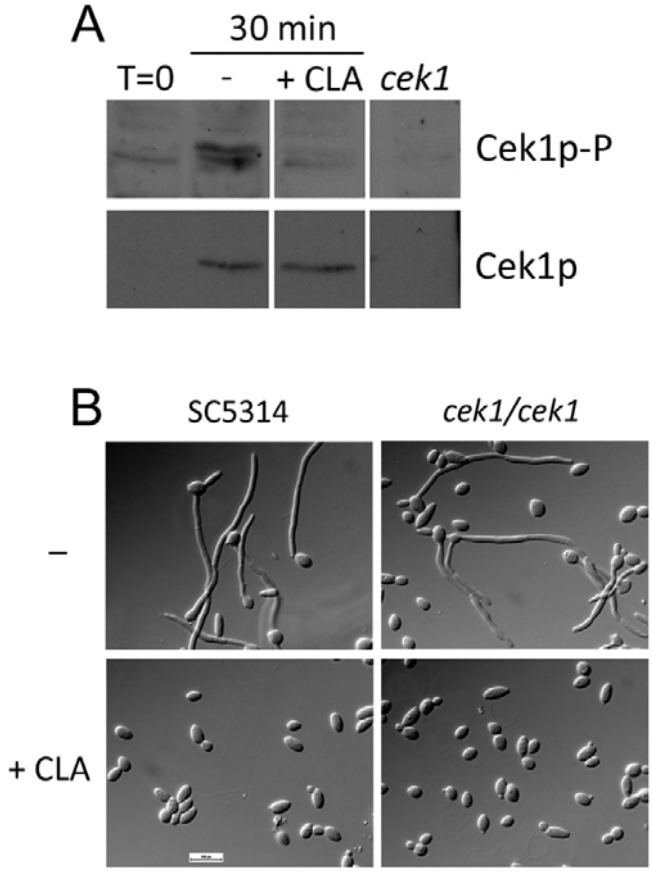

Figure 4. 7 CLA affects the activation of the MAP kinase pathway.

(A) CLA represses Cek1p phosphorylation. Total protein extracts were prepared from SC5314 and *cek1/cek1* strains grown in Spider medium at 37°C for the indicated times, in the absence or presence of 100 μM CLA. The phosphorylated form of Cek1p was detected using anti-p44/p42 antibodies (indicated as Cek1p-P), while total cellular levels of Cek1p were detected using a rabbit anti-Cek1p. (B) *CEK1* is not required for CLA-mediated inhibition of hyphal growth. SC5314 and *cek1/cek1* strains were grown as described in (A) for 4 h. Cells were visualized using DIC optics at ×100 magnification. Bar = 10 μm.

Cellular cAMP concentrations increase as a result of the binding of Ras1p to Cyr1p. Thus, lower cAMP levels may indicate that CLA disrupts the Ras1p-Cyr1p branch of Ras1p signaling. Instead of measuring cAMP concentrations directly, the behavior of cAMP responsive genes was examined in presence of CLA. Transcriptional profiles of CLA-treated cells and a *cyr1/cyr1* mutant strain lacking cAMP did not overlap, suggesting cAMP concentrations were not reduced in CLA-treated cells (data not shown). Several reasons may account for this result. First, CLA's effect on cAMP levels may be more subtle than effects resulting from the deletion of *CYR1*. Indeed, cAMP-downregulated genes such as *CSP37*, *BCY1*, *TPK2*, *SOD2*, and *HSP12* (Bahn *et al.*, 2007; Harcus *et al.*, 2004) were somewhat upregulated in CLA-treated cells (Figure 4.8A). Second, CLA may affect cAMP levels prior the 90-minute time point at which gene expression analyses were performed, given that the spike in cAMP levels is transient and rather short-lived (Jung & Stateva, 2003; Zhu *et al.*, 2009). Third, cAMP synthesis may simply be Ras1p-independent, and thus not affected by CLA. In *S. cerevisiae*, cAMP production is stimulated by Cyr1p interacting with Gpa2p (Kubler *et al.*, 1997; Lorenz & Heitman, 1997), while in *C. albicans*, Gpr1p-Gpa2p function upstream of Cyr1p (Maidan *et al*., 2005a). Additionally, Ras1p does not signal exclusively through Cyr1p, as the deletion of *RAS1* only affects a subset of Cyr1p-dependent genes (Harcus *et al.*, 2004). Hence, CLA may be affecting Ras1p signaling independently of Cyr1p and cAMP signaling. To determine whether or not CLA impacts Ras1p-cAMP branch of Ras1p signaling, the levels of Ras1p interacting with Cyr1p in untreated and CLA-treated cells could be examined.

If CLA blocked filamentation by interfering with the association of Ras1p to Cyr1p and the synthesis of cAMP, then the addition of exogenous cAMP should restore proper cAMP levels as well as hyphal growth to CLA-treated cultures. Providing exogenous cAMP to correct hyphal defects is a standard way to demonstrate that a mutation or a molecule impacts cAMP signaling in *C. albicans* (Davis-Hanna *et al.*, 2008; Leberer *et al.*, 2001). When CLA-treated cultures were amended with 10 mM dibutyryl cAMP, filamentation was only restored partially (Figure 4.8B). These results, combined to the fact

cAMP-responsive genes were not modulated by CLA, may indicate that CLA affects Ras1p signaling, but not cAMP signaling. However, it seems that using exogenous cAMP to rescue hyphal defects may yield inconsistent results. For instance, one group showed that exogenous cAMP rescued the filamentation defect of a *cyr1/cyr1* mutant (Leberer *et al.*, 2001; Rocha *et al.*, 2001), while another group reported that no more than 5% of the *cyr1/cyr1* cells exhibited hyphal growth (Fang & Wang, 2006).

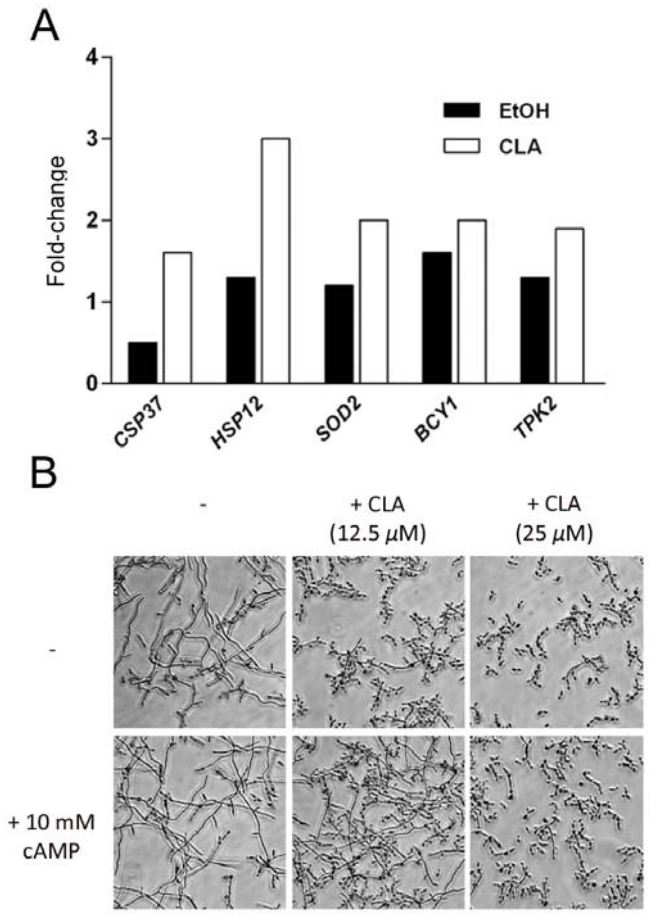

Figure 4. 8 CLA's effect on cAMP signaling

(A) Fold-change of several cAMP-downregulated genes in untreated and CLA-treated cells at 37°C. The significantly differentially expressed genes were obtained by comparing the transcriptional profile of cells at 37°C with that of CLA-treated cells at 37°C and are listed in Table S4. Data are fold-change values. (B) Effects of dibutyryl cAMP (10 mM) on SC5314 cells grown in Spider medium in the absence or presence of CLA for 5 h at 37°C. Cells were visualized at ×40 magnification.

Upon binding cAMP, Bcy1p releases the catalytic subunits of PKA, thereby enabling their activity. Instead of examining CLA's effect on PKA activity, the role played by Tpk1p or Tpk2p in the CLA-mediated hyphal growth inhibition was determined. In liquid Spider medium, filamentation depended mostly on *TPK2*. However, CLA inhibited the residual filamentous growth in the *tpk2/tpk2* mutant strain, suggesting that *TPK2* was not required for CLA's effect on filamentation (Figure 4.9). Similar results were obtained using the *tpk1/tpk1* mutant and the *PCK1p-TPK1/tpk1/tpk2/tpk2* strain, in which both *TPK2* alleles are missing and one of the *TPK1* alleles is placed under the control of the *PCK1* promoter (data not shown). The conditional mutant was reported to exhibit a severe filamentation defect in Spider medium (Bockmuhl *et al.*, 2001). In our hands, it behaved similarly to the *tpk2/tpk2* mutant, probably because the *PCK1* promoter was moderately expressed in Spider medium. That CLA inhibited hyphal growth independently of *TPK1* and *TPK2* may be due to the functional redundancy exhibited by both PKA isoforms.

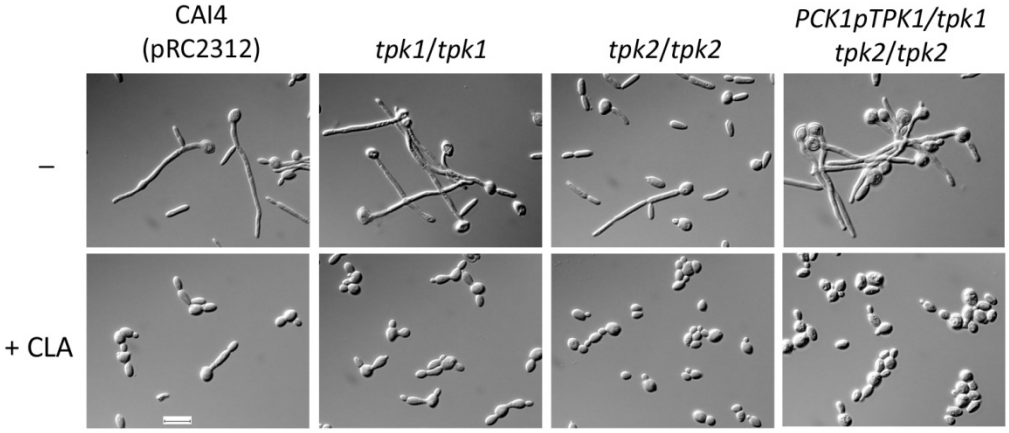

Figure 4. 9 Morphology of PKA mutant strains.

Parental and mutant strains *tpk1/tpk1* (IIHB6), *tpk2/tpk2* (TPO7.4), and *PCK1pTPK1/tpk1/tpk2/tpk2* (M231) were induced to filament in Spider medium at 37°C in the absence or presence of CLA (100 μM) for 4 h. Cells were visualized by DIC optics at ×100 magnification. Bar = 10 μm.

The morphogenetic regulator Efg1p is necessary for hyphal growth in most hypha-inducing conditions, including in Spider medium (data not shown). Thus, Efg1p was believed to be a potential downstream target of CLA. However, several lines of evidence clearly demonstrate the opposite, in that CLA does not affect the Efg1p branch of Ras1p signaling. First, CLA had no effect on *EFG1* mRNA or protein levels, as both decreased in untreated and CLA-treated cells (Figure 4.10A and inset). Second, several glycolytic genes, including *FBA1*, *PGK1*, and *GLK1*, which are induced in an *EFG1*-dependent manner were not modulated by CLA (Figure 4.10B) (Doedt *et al*., 2004). Third, *TEC1* expression levels were downregulated by CLA independently of *EFG1* (Figure 4.10C). The Efg1p-independent modulation of Ras1p signaling has rarely been reported, except in the case of geldanamycin-induced filamentation (discussed in section 3.8). The Hsp90p inhibitor geldanamycin was shown to promote filamentous growth by modulating the Ras1p-cAMP-PKA signaling pathway independently of Efg1p (Shapiro *et al.*, 2009).

Taken together, results presented in Chapter 2 suggest that CLA reduces Ras1p cellular levels as well as *TEC1* mRNA levels, without affecting cAMP signaling or Efg1p levels and activity. At first glance, it appears difficult to reconcile such results with the current Ras1p signaling model, which proposes that Ras1p interacts with Cyr1p to produce cAMP, which activates PKA and Efg1p. Fortunately, two lines of evidence concur with our findings, the first being that Ras1p does not only signal through Cyr1p (Harcus *et al*., 2004), the second being the existence of a Ras1p-dependent pathway parallel to the Efg1p and MAP kinase pathways, which may include Tpk1p or Tpk2p, but not Efg1p (Bockmuhl *et al*., 2001). The latter conclusion was reached based on the fact that *RAS1* overexpression suppressed the hyphal growth defect of an *efg1/efg1* mutant, but not of *tpk1/tpk1* and *tpk2/tpk2* mutants. Thus, it is possible that CLA's effect on Ras1p signaling may be mediated by Ras1p, PKA and Tec1p, independently of Cyr1p, cAMP, and Efg1p. CLA may affect *TEC1* expression either via an unknown regulator or via PKA.

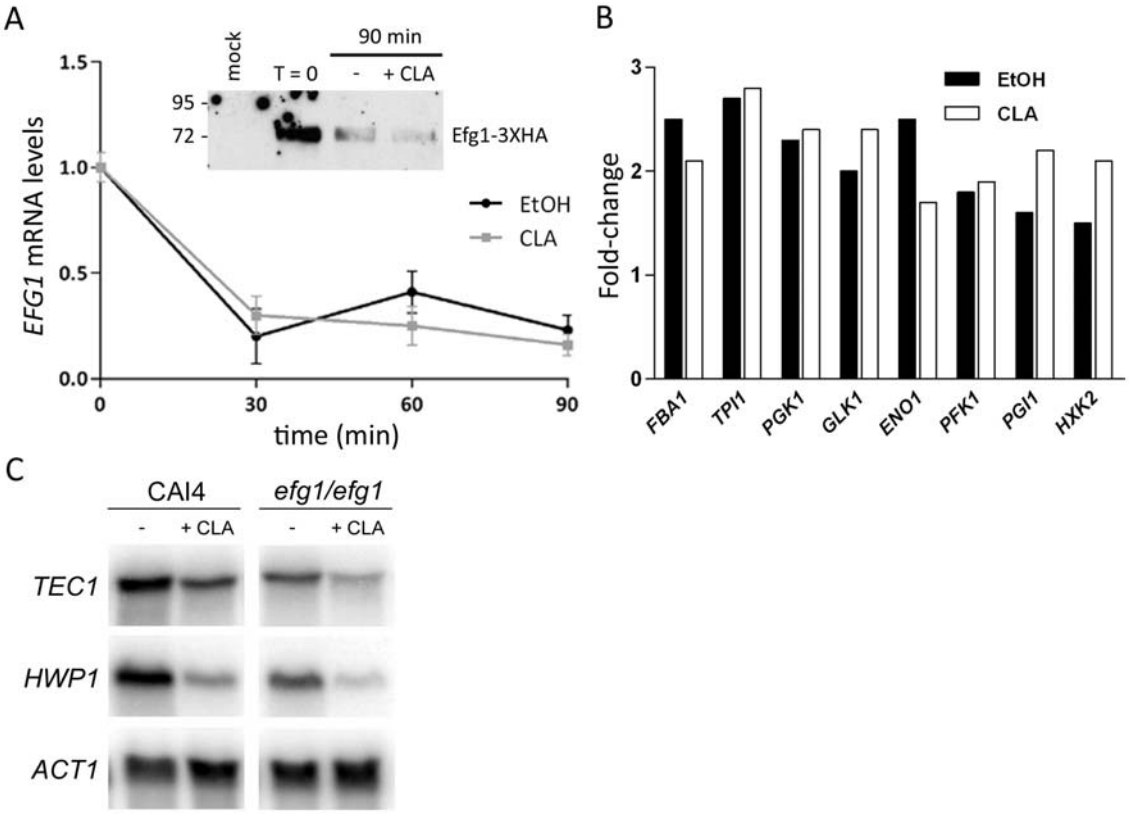

Figure 4. 10 CLA does not modulate the Efg1p-dependent branch of Ras1p signaling.

(A) *EFG1* expression levels in cells induced to filament in Spider medium in the absence or presence of CLA. Transcript levels of *EFG1* were measured by quantitative PCR and normalized to *ACT1*. Relative expression levels were obtained by normalizing data for each time point to data obtained at time zero. Data are means and standard deviations for duplicate biological samples. (inset) Total protein extracts were prepared from SC5314 cells (mock) and Efg1p-3XHA (JS2284) cells at the zero time point or grown as in (A) for 90 minutes. Efg1p-3XHA was immunoprecipitated using Anti-HA Affinity Matrix. Western blotting analysis was performed using anti-HA antibodies (clone 12CA5). Molecular masses are indicated on the left. (B) Expression levels of Efg1p-regulated glycolytic genes. Data are fold-change values and were obtained by comparing growth in Spider medium at 37°C in absence or presence of CLA to that at 30°C. (C) *TEC1* and *HWP1* expression levels in parental and *efg1/efg1* (HLC52) strains grown as described in (A) for 90 minutes. *ACT1* is shown as the loading control.

4.4 CLA affects the Tup1p-Nrg1p signaling pathway

Because the hyphal growth program is controlled by a network of signaling pathways, the effect of CLA can be expected to be multifactorial, in that the fatty acid may affect more than one signaling pathway. Other small molecules that modulate hyphal growth in *C. albicans* have been shown to have more than one target. For instance, the Tor1p specific inhibitor rapamycin enhanced filamentation and induced cellular aggregation by downregulating *NRG1* and *TUP1* expression and by activating transactivators Efg1p and Bcr1p (Bastidas *et al*., 2009) (discussed in section 3.7). Farnesol inhibited hyphal growth by affecting at least three different signaling pathways, including the Ras1p-cAMP-PKA pathway (Davis-Hanna *et al*., 2008), both the Hog1p and the Cek1p MAP kinase pathways (Roman *et al.*, 2009a; Smith *et al.*, 2004), and two regulators, namely the transcription factor Tup1p (Kebaara *et al*., 2008) and the histidine kinase Chk1p (Kruppa *et al*., 2004) (discussed in section 3.2). In addition to targeting the Ras1p signaling pathway, CLA appears to modulate the Tup1p- Nrg1p pathway. Indeed, transcriptional profiling showed that CLA downregulated several Tup1p- and/or Nrg1p-dependent genes, suggesting CLA may affect the Tup1p-Nrg1p signaling pathway (Figure 4.11). Both *TUP1* and *NRG1* were required for CLA to inhibit filamentation (Figure 2.7A). Additionally, CLA prevented the downregulation of *TUP1* and *NRG1* transcripts, thus hindering the relief of *TUP1* repression, which is required for filamentation to proceed (Figure 2.7B). Taken together, these results strongly suggest that the Tup1p-Nrg1p signaling pathway is involved in mediating CLA's hypha-inhibiting properties.

Although Tup1p-mediated repression plays a major role in the yeast-to-hypha transition in *C. albicans*, upstream regulators of the signaling pathway have yet to be identified (Hall *et al.*, 2009; Hogan & Sundstrom, 2009). Braun & Johnson (2000) established that Tup1p, Cph1p, and Efg1p belonged to different signaling pathways and made independent contributions to the hyphal growth program. However, several lines of evidence argue against the rigidity of this model. First, *RAS1* is induced upon the yeast-to-hypha transition independently of Tup1p, Nrg1p, and Rfg1p (Kadosh & Johnson, 2005),

suggesting that *RAS1* lies upstream of/parallel to Tup1p, Nrg1p, and Rfg1p. Second, the downregulation of *NRG1* transcript levels depends on *EFG1* (Braun *et al*., 2001). Moreover, *EFG1*, *TEC1*, *CPH1*, and *CPH2* are required for the decrease in Nrg1p protein levels, thereby suggesting Nrg1p is a downstream effector of these transcription factors (H. Liu, unpublished results). Third, components of the MAP kinase and cAMP-PKA signaling pathways, including *CST20*, *CEK1*, *CPH1*, and *PDE2*, are either Tup1p- and/or Nrg1p-regulated, suggesting that the relief of Tup1p repression exerts feedback on pathways that positively regulate the hyphal growth program (Jung & Stateva, 2003; Murad *et al.*, 2001a; Murad *et al.*, 2001b).

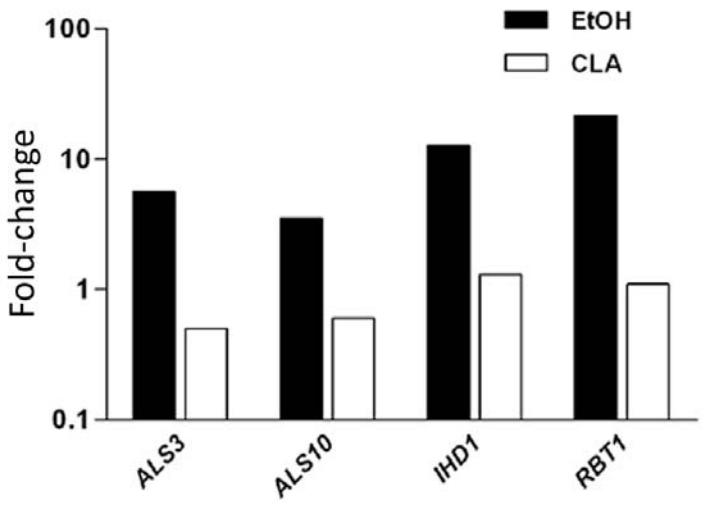

Figure 4. 11 CLA affects the expression of Tup1p-Nrg1p-regulated genes.

Fold-change of genes regulated by the Tup1p-Nrg1p repressor complex in untreated and CLA-treated cells at 37°C. The significantly differentially expressed genes were obtained by comparing the transcriptional profile of cells at 37°C with that of CLA-treated cells at 37°C and are listed in Table S4. Data are fold-change values.

The relief of Tup1p-dependent repression may occur as a result of the Ras1p-cAMP-PKA-Efg1p signaling pathway being activated. Activation of Efg1p and increased Tec1p levels may result in the downregulation of *NRG1* mRNA and protein levels, which allows the Tup1p-mediated repression to be lifted. Thus, if Tup1p signaling is truly regulated by Ras1p signaling, then CLA's effect on *TUP1* and *NRG1* expression was indirect, resulting instead from the fatty acid affecting Ras1p signaling. This suggestion is speculative, given that the upstream regulators of the Tup1p-Nrg1p pathway have not been identified.

4.5 Proposed mechanism of action of CLA

One has to bear in mind that in addition to modulating GFP-Ras1p cellular levels, CLA also affected *RAS1* transcript levels (Figure 2.6A), raising a question: are these events independent from one another? If not, which one occurs first? The precise mechanism by which hypha-inducing signals trigger Ras1p-mediated hyphal growth are not known yet (Hogan & Sundstrom, 2009). Additionally, regulators/inducers of *RAS1* expression have not been identified in *C. albicans*. However, in *S. cerevisiae*, the regulatory subunit of PKA, Bcy1p, seems to be involved in the upregulation of *RAS2* expression in response to growth at 37°C (Gasch *et al.*, 2000; Segal *et al.*, 2003).

One may imagine that in response to hypha-inducing conditions, membrane-associated Ras1p is GTP-loaded and activated. In turn, the cAMP-PKA and the MAP kinase signaling pathways are activated, inducing the hyphal growth program and promoting filamentation. The activated hyphal growth program may somehow engage a specific regulator of *RAS1* (Bcy1p?), resulting in increased *RAS1* mRNA and protein levels. A pool of newly synthesized and lipid-modified Ras1p may then be targeted to the membrane, thus sustaining Ras1p signaling and maintaining the hyphal growth program activated. Additionally, the downstream effectors of Ras1p signaling Efg1p and Tec1p may downregulate *NRG1* expression, resulting in the relief of Tup1p repression. The control of

RAS1 transcription by Ras1p signaling would generate a positive feedback loop, thereby ensuring an increased pool of membrane-associated Ras1p molecules and driving Ras1p signaling, the hyphal growth program, and filamentation.

According to the aforementioned sequence of events regulating filamentation, it is possible to foresee at which point CLA may interfere with the hyphal growth program. First, it should be mentioned that CLA does not affect Ras1p signaling and the hyphal growth program instantaneously. Indeed, Tec1p protein levels were induced at the 30 minute time point in CLA-treated cells, suggesting that the hyphal growth program was initially activated, even in presence of CLA (Figure 4.12). This result somewhat correlates with the finding that cAMP levels were not significantly modulated by CLA, as Ras1p-cAMP signaling may have been initially induced, despite the presence of CLA (discussed in 4.3.5.2). Thus, in CLA-treated cells, Ras1p signaling is initially activated. Concomitantly, Ras1p depalmitoylation/palmitoylation cycles may force Ras1p to leave the plasma membrane, to be relocalized and repalmitoylated at other intracellular membranes, and to be retargeted to the membrane. Meanwhile, CLA may be incorporated into membrane phospholipids, thus altering the membrane's unsaturation levels and structure. CLA may affect Ras1p signaling at this point: repalmitoylated Ras1p may not be able to associate stably with the unsaturated membrane due to the weakening of van der Waals interactions between anchors of lipidated proteins and phospholipid acyl chains (Seo *et al*., 2006) (discussed in sections 4.3.3; 4.3.4)

Consequently, reduced membrane-bound Ras1p levels may downregulate Ras1p signaling, which impairs the activation of the MAP kinase pathway, downregulates *TEC1* expression (mRNA and protein levels), and the overall hyphal growth program. Additionally, decreased Tec1p levels may prevent the downregulation of *NRG1* and the relief of Tup1p-mediated repression. In turn, the *RAS1* regulator (Bcy1p?) may no longer be actively engaged in the hyphal growth program, preventing the increase in *RAS1* mRNA and protein levels, and abrogating the positive feedback exerted on Ras1p signaling. In

turn, levels of membrane-bound Ras1p continue to decrease, which further enhances the impairment of Ras1p signaling and the downregulation of the hyphal growth program. This unifying model takes into account all results pertaining to the effect of CLA on molecular events that regulate the yeast-to-hypha transition in *C. albicans*. However, it holds true until the regulators of *RAS1* and the mechanism by which Ras1p signaling is induced are identified.

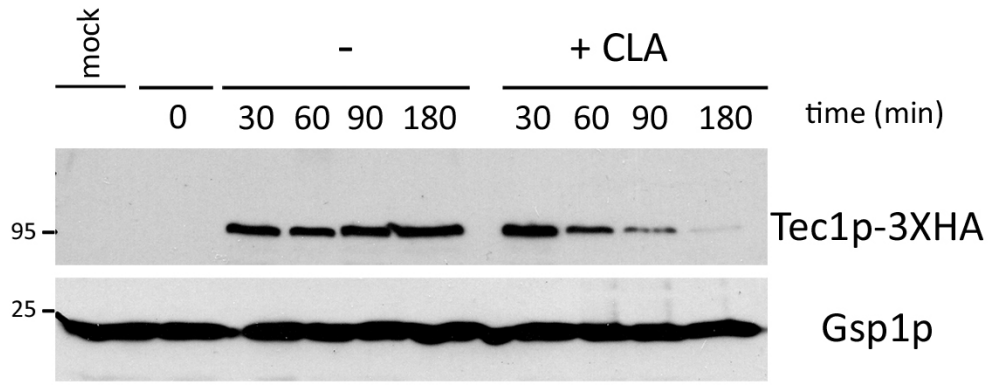

Figure 4. 12 Tec1p-3XHA protein levels are reduced in presence of CLA.

Tec1p protein levels were analyzed using a strain expressing Tec1p-3XHA. Total protein extracts were prepared from SC5314 (mock) and Tec1p-3XHA strains induced to filament in Spider medium at 37°C supplemented or not with CLA. Western blotting analysis was performed using anti-HA antibodies. Gsp1p, shown as a loading control, was detected using antibodies raised against *S. cerevisiae* Gsp1p. Molecular weights (kDa) are shown on the left.

4.6 Therapeutic potential of molecules that modulate morphogenesis in *C. albicans*

4.6.1 Modulating morphogenesis as a means to treat candidiasis

Impairing morphogenesis of *C. albicans* has been proposed as a means to treat candidiasis. Indeed, using engineered strains in which the yeast-to-hypha transition was manipulated externally, blocking the morphogenetic transition reduced virulence in a model of systemic candidiasis (Saville *et al.*, 2003; Saville *et al.*, 2006). These findings reinforced the view that the ability to switch from one growth form to another is an important virulence attribute of the pathogenic yeast and constitutes a target for the development of antifungal drugs (Lo *et al.*, 1997; Zheng & Wang, 2004). In parallel, many small molecules have been reported to modulate morphogenesis in *C. albicans*, but very few have been evaluated for their therapeutic properties in the context of infection (reviewed in Chapter 3). So far, farnesol appears to be the only morphogenesis-modulating molecule used successfully to treat *Candida* infections. However, it was effective only against mucosal candidiasis, as it increased virulence in a model of systemic candidiasis (Hisajima *et al.*, 2008; Navarathna *et al.*, 2007). The histone deacetylase inhibitor trichostatin A (TSA) is another morphogenesis-modulating molecule that may have therapeutic potential, although it has yet to be evaluated in vivo. TSA may prove useful for treating systemic candidiasis, as the deletion of its target (*SET3*) attenuated virulence in a mouse model of systemic candidiasis (Hnisz *et al.*, 2010).

Interfering with the morphogenetic transition of *C. albicans* to treat candidiasis is in line with the current trend in antifungal drug development, which favors targeting virulence processes instead of essential processes (Gauwerky *et al.*, 2009; Jiang *et al.*, 2002). Theoretically, such a strategy does constitute a sound therapeutic approach. Additionally, many small molecules that impair the yeast-to-hypha transition have been identified and characterized. However, as discussed in Chapter 3, an overview of the literature revealed that few of these molecules have been tested in vivo. Due to an important lack of clinical

evidence, modulating morphogenesis in *C. albicans* as a means to treat candidiasis remains a questionable therapeutic approach. Until the therapeutic potential of small molecules is evaluated in the context of different *Candida* infections, it can only be speculated that such compounds can be used to treat candidiasis.

4.6.2 Putative therapeutic applications of CLA and fatty acids

CLA's effect on hyphal growth and virulence has not been tested in the context of candidiasis. However, based on previous evidence, on the nature of fatty acids, and on the type of *Candida* infection, several therapeutic applications of CLA and fatty acids may be suggested. One application of fatty acids could be to incorporate them into materials used to produce medical implants. Indwelling medical devices such as catheters, pacemakers, and prosthetic joints often become colonized with *C. albicans* biofilms, resulting in failure of the implant and a localized infection that can disseminate (Nobile & Mitchell, 2006; Ramage *et al.*, 2009). Medical devices made of materials containing and/or surface-coated with fatty acids may thwart the development of *C. albicans* biofilms by preventing filamentation. There is a precedent to this type of application. Undecylenic acid present in denture liners inhibited the yeast-to-hypha switch in vitro (McLain *et al*., 2000). Although its presence in denture liners stemmed from serendipity rather than from scientific evidence, undecylenic acid was proposed to inhibit hyphal growth of *C. albicans* and prevent denture stomatitis, a condition often associated with *Candida* infections. CLA and other fatty acids may be expected to perform similarly.

Topical formulations containing fatty acids could also be developed to treat superficial candidiasis such as oral thrush or VVC, as has been proposed for acetylenic acids and farnesol (Li *et al.*, 2008; Navarathna *et al.*, 2007). For instance, vaginal gels containing glycerol monolaurate, a 12-carbon fatty acid monoester, were shown to inhibit *C. albicans* and were well tolerated in patients (Strandberg *et al.*, 2010).

Dairy-based, fatty acid-enriched probiotic products containing CLA and/or other fatty acids could be developed to prevent and/or GI candidiasis and VVC. GI candidiasis and VVC are due to the overgrowth of *C. albicans*. Probiotic microorganisms produce fatty acids, such as butyric and capric acids, which exert hypha-inhibiting properties (Krasowska *et al.*, 2009; Murzyn *et al.*, 2010; Noverr & Huffnagle, 2004). Additionally, fatty acids are present in milk (German & Dillard, 2006). However, the therapeutic potential of such a product would not only stem from the hypha-inhibiting fatty acids present in milk or produced by probiotic microorganisms, but also from the microorganisms themselves, which compete with *C. albicans* for nutrients and for attachment sites on host cells, and inhibit growth of the pathogenic yeast (Hogan & Kolter, 2007). Probiotic formulations have been used successfully to protect from *Candida* infections (Elahi *et al.*, 2005; Hilton *et al.*, 1992; Martinez *et al.*, 2009).

Fatty acids could also be administered as adjuncts to conventional antifungal drugs. Indeed, an ongoing trend in antifungal therapy is the use of new combination therapies to enhance the efficacy of currently used drugs and to limit the evolution of antifungal resistance (Cowen & Steinbach, 2008; Steinbach *et al.*, 2007). For instance, Hsp90p or calcineurin inhibitors used in combination with classical antifungals enhanced the activity of conventional antifungal agents and improved the treatment of various fungal diseases (Cowen *et al.*, 2009; Steinbach *et al.*, 2007). Similarly, arachidonic acid increased the antifungal susceptibility of *C. albicans* to amphotericin B and clotrimazole (Ells *et al.*, 2009). Thus, it is possible to imagine a similar application for CLA and other fatty acids.

4.7 Ecological role of fatty acids and other quorum sensing molecules in *C. albicans* morphogenesis

Hypha-inhibiting fatty acids, including CLA, were initially isolated from bovine whey (Clement *et al*., 2007). Although interesting, these findings do not quite fit with the ecology of *C. albicans*, whose primary environmental niche is generally considered to be the intestinal tract of warm-blooded animals (Odds, 1988a). Additionally, it is rather unlikely to find *C. albicans* in milk or in dairy products (i.e. whey). So, is there a natural environment in which *C. albicans* is exposed to fatty acids, thus underpinning their effect on the morphogenetic transition of the pathogenic yeast? Or were these findings merely anecdotal? In the following section, the ecological significance of the hypha-inhibiting properties of fatty acids and other quorum sensing molecules is discussed.

C. albicans inhabits the mucocutaneous surfaces of the oral cavity, the GI tract, and the vaginal cavity. In these host niches, the pathogenic yeast is bound to encounter microorganisms of the endogenous microflora. Interestingly, several lines of evidence show that many bacteria and yeast interact with *C. albicans* via fatty acid-like signaling molecules. For instance, the oral pathogen *Streptococcus mutans* was shown to secrete a fatty acid signaling molecule, trans-2-decenoic acid, which inhibited the yeast-to-hypha transition (Vilchez *et al.*, 2010). This compound is structurally similar to the bacterially-secreted diffusible signal factor (DSF) and to farnesoic acid, and has been termed *Streptococcus* DSF (SDSF) (Oh *et al.*, 2001; Wang *et al.*, 2004). Culture supernatants of lactic acid bacteria (LAB), important inhabitants of the gut and female reproductive tract, were shown to block germ tube formation (Noverr & Huffnagle, 2004). This effect was mimicked by the presence of 25 mM butyric acid, a concentration well within the physiological range observed in the colon (Noverr & Huffnagle, 2004; Saemann *et al.*, 2002). LAB produce biologically active short chain fatty acids as by-products of anaerobic fermentation, which may affect *C. albicans* morphogenesis (Saemann *et al.*, 2002). Similarly, the probiotic yeast *Saccharomyces boulardii* inhibited the yeast-to-hypha

transition, adhesion, and biofilm formation of *C. albicans* by secreting capric acid (Krasowska *et al.*, 2009; Murzyn *et al.*, 2010). *P. aeruginosa* secretes the 3-oxo-C12 acyl homoserine lactone, while *B. cenocepacia* produces cis-2-dodecenoic acid (BDSF), two molecules that inhibit the yeast-to-hypha transition (Boon *et al.*, 2008; Hogan *et al.*, 2004). These quorum sensing molecules are structurally similar to the hypha-inhibiting molecules farnesoic acid and cis-11-methyl-2-dodecenoic acid (DSF) secreted by *C. albicans* and *Xanthomonas campestris*, respectively (Oh *et al.*, 2001; Wang *et al.*, 2004).

C. albicans is also bound to encounter fatty acids derived from host cells, e.g. in macrophages and at the surface of the skin. Gene expression analysis demonstrated that upon phagocytosis, *C. albicans* cells underwent drastic metabolic changes, switching to an alternative carbon metabolism (Lorenz *et al*., 2004). These results suggested that cells were exposed to fatty acids, such as arachidonic acid (AA), derived either from *Candida* itself or from the internal milieu of the macrophage. Phagocytosed cells eventually formed hyphae, despite the presence of AA whose concentration may have been too low to block morphogenesis. Conditions found on the skin, which produces surface oils, promoted germ tube formation, white-opaque switching, and mating in *C. albicans* (Lachke *et al.*, 2003). While conditions of the skin were not examined *per se*, oxylipins were suggested to play a role in these developmental processes (Erb-Downward & Huffnagle, 2006; Noverr & Huffnagle, 2004). In addition, opaque cells are known to have an oxidative metabolism, which may be indirect evidence that opaque cells present at the surface of the skin are exposed to fatty acids (Lan *et al.*, 2002).

In several of its preferred niches, *C. albicans* encounters fatty acids or fatty acid-like molecules, either produced by members of the host microbiota or derived from host cells, which generally inhibit its hyphal development. The secretion of hypha-inhibiting compounds may be a means by which antagonistic microorganisms maintain *C. albicans* in the yeast form and reduce its attachment to host surfaces, providing them with a competitive edge over the pathogenic yeast. Interactions between *C. albicans* and other

microorganisms mediated by fatty acid-like molecules appear to be ubiquitous. Given that milk is not a natural habitat of *C. albicans*, the discovery that whey-derived fatty acids inhibited hyphal growth was serendipitous. Yet, bacteria, yeast, and host cells interact with *C. albicans* in its natural niches by secreting fatty acids and lipidic quorum sensing molecules. Thus, the inhibitory effects of fatty acids and fatty acid derivatives on hyphal growth in *C. albicans* appear to be ecologically significant.

Conclusion

This study has allowed the hypha-inhibiting effects of several fatty acids to be characterized and the mechanism by which CLA blocked the yeast-to-hypha transition to be deciphered. Inhibition of hyphal growth stemmed from CLA (i) reducing levels of membrane-bound Ras1p and impairing Ras1p signaling, possibly by altering the plasma membrane structure, (ii) preventing the activation of the MAP kinase pathway, (iii) downregulating the PKA-*TEC1* branch of Ras1p signaling, (iv) inhibiting the increase in *RAS1* mRNA and protein levels, (v) possibly blocking the positive feedback loop on Ras1p signaling, and (vi) preventing the relief of the Tup1-mediated repression of filamentation. While it is possible to speculate that all fatty acids have the capacity to affect Ras1p signaling, it remains that the findings presented in this work are specific to CLA. Ras1p signaling controls aspects of filamentation and virulence in other fungi. Moreover, CLA inhibited hyphal growth in other *Candida* species, as well as in other filamentous fungi. Thus, it is tempting to suggest that CLA possesses a broad spectrum of anti-filamentation activity, given that its target is ubiquitous. However, the effect of CLA on Ras1p signaling in other fungi should also be demonstrated.

Elucidating the mechanism of action of CLA had another purpose, namely to identify CLA's target(s) and predict whether or not the fatty acid would have deleterious effects in humans, if it were to ever be used clinically. CLA affects Ras1p, a protein found in humans, and is thus bound to affect mammalian Ras proteins. However, this effect is unlikely to be a harmful one. Indeed, other PUFAs have been reported to modulate mammalian Ras proteins, and have had beneficial effects, namely by reducing tumorigenesis (Chapkin *et al.*, 2008; Collett *et al.*, 2001).

This work may constitute the rationale to evaluate CLA's therapeutic potential in the context of *Candida* infections. The therapeutic properties of CLA and other small morphogenesis-modulating molecules should be assessed to remedy the lack of clinical evidence demonstrating that impairing the yeast-to-hypha transition in *C. albicans* is a means to treat candidiasis. In addition, because such molecules target a virulence

determinant rather than an essential process, they are in line with the current trend in antifungal drug development. Studies evaluating the therapeutic potential of small molecules such as CLA to treat *Candida* infections are warranted, and may lead to the identification of new antifungal agents.

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Appendix

Chapter 2. Supplementary tables (Table S1 and S4)

Declaration of coauthors

Authorization of editor

Curriculum vitae

Supplementary Table S1. The significantly differentially expressed obtained by comparing "Experiment 37°C" with itself. Differentially expressed genes were defined by a fold difference > 2 and a P-value < 0.05. Only upregulated genes are shown.

Systematic	P-value	Norm	Norm	Norm	Name	
		37°C	30°C + CLA	37°C + CLA	520 genes	Y→H (Lee)
orf19.3374	4,14E-04	22,6	0,4	0,7	ECE1	√
orf19.1327	2,04E-03	21,4	0,3	1,1	RBT1	√
orf19.5760	1,22E-04	12,6	0,2	1,3	IHD1	√
orf19.2060	5,46E-04	10,9	0,2	0,2	SOD5	√
orf19.2020	1,71E-03	8,8	1,0	2,8	HGT6	
orf19.2210	1,94E-03	8,2	1,1	1,5		
orf19.1321	5,76E-03	7,8	0,4	0,9	HWP1	√
orf19.815	4,86E-03	6,4	0,9	1,2	DCK1	√
orf19.7668	4,31E-03	5,9	1,4	3,3	MAL2	
orf19.3384	1,41E-02	5,7	0,3	0,5		
orf19.1816	3,91E-03	5,6	0,3	0,5	ALS3	√
orf19.701	9,68E-04	5,6	0,6	0,6	CFL11	√
orf19.2877	1,12E-04	5,0	0,7	3,7	PDC11	√
orf19.5585	1,88E-03	5,0	1,4	1,6	SAP5	
orf19.675	4,50E-02	4,9	2,1	1,8		
orf19.6387	1,65E-02	4,8	1,4	3,6	HSP104	
orf19.1354	3,13E-02	4,7	0,7	2,1	UCF1	
orf19.6028	6,89E-05	4,6	0,7	0,8	HGC1	√
orf19.3707	2,22E-03	4,2	1,1	2,7	YHB1	
orf19.5114.1	6,68E-03	4,0	0,6	2,6		
orf19.4980	8,95E-03	4,0	1,2	3,6	HSP70	
orf19.918	4,78E-03	3,7	1,2	3,2	CDR11	√
orf19.7323	5,04E-03	3,6	1,2	2,8	CBP1	√
orf19.7085	2,30E-02	3,6	1,0	2,4		
orf19.2737	3,13E-04	3,6	2,5	3,3		
orf19.2770.1	2,99E-05	3,6	1,7	4,6	SOD1	√
orf19.4450.1	3,58E-02	3,6	0,5	1,5		
orf19.2355	7,66E-03	3,5	0,5	0,6	ALS10	
orf19.5102	6,68E-03	3,5	1,6	1,9	PLB5	√

orf19.3981	3,94E-06	3,5	0,7	0,6	MAL31	
orf19.7350	4,15E-03	3,5	2,3	2,8		
orf19.1715	1,92E-04	3,4	1,2	1,3	IRO1	
orf19.6169	1,50E-02	3,4	0,6	0,8		
orf19.1490	1,27E-03	3,4	1,1	1,4	MSB2	√
orf19.5636	6,91E-03	3,4	1,3	1,7	RBT5	√
orf19.3997	7,13E-03	3,3	0,9	3,1	ADH1	√
orf19.7676	6,78E-03	3,3	1,6	2,4	XYL2	
orf19.2292	3,28E-08	3,3	0,8	0,8		
orf19.1161	1,52E-03	3,3	1,3	1,8	PLD1	
orf19.4527	3,95E-02	3,3	1,1	1,4	HGT1	√
orf19.2833	6,14E-03	3,3	0,8	1,2	PGA34	
orf19.638	3,32E-04	3,3	1,7	1,8	FDH1	
orf19.1847	2,51E-04	3,2	1,7	3,0	ARO10	√
orf19.3823	1,14E-02	3,2	1,1	1,5	ZDS1	√
orf19.6928	3,80E-02	3,2	2,2	2,1	SAP9	
orf19.3746	1,31E-02	3,2	1,0	1,1	IFC1	
orf19.6229	1,34E-02	3,2	8,7	9,4	CAT1	
orf19.1799	8,31E-05	3,1	1,1	1,2		
orf19.14	3,10E-02	3,1	1,7	1,7		
orf19.2151	6,22E-03	3,1	1,4	2,3	NAG6	√
orf19.6202	2,09E-02	3,1	2,4	2,2	RBT4	√
orf19.1407	3,24E-02	3,1	1,9	1,7		
orf19.3718	3,63E-03	3,1	0,9	0,9		
orf19.1585	7,26E-03	3,1	1,5	1,6	ZRT2	
orf19.1187	1,78E-03	3,0	1,3	2,5	CPH2	
orf19.2685	8,19E-03	2,9	0,8	1,4	PGA54	
orf19.6844	4,25E-02	2,9	5,2	3,6	ICL1	
orf19.4732	5,73E-04	2,9	2,0	3,0	SEC24	√
orf19.6408	1,71E-02	2,9	1,9	1,9		
orf19.6705	2,57E-02	2,9	0,9	1,0		
orf19.6114	1,83E-02	2,9	1,4	2,4		
orf19.7021	1,17E-05	2,8	1,0	1,6	GPH1	
orf19.4056	4,57E-02	2,8	0,8	1,0	GAT2	√
orf19.449	1,52E-02	2,8	1,8	2,1		
orf19.2170	1,24E-02	2,8	0,9	1,2	PHM7	
orf19.2777	6,82E-03	2,8	1,3	1,4		
orf19.6745	1,33E-04	2,7	1,7	2,8	TPI1	
orf19.6287	9,49E-03	2,7	2,1	1,5	AAT21	√

orf19.5447	1,02E-02	2,7	0,8	1,1	HGT19	
orf19.6178	5,40E-03	2,7	2,6	2,7	FBP1	√
orf19.5908	2,47E-02	2,7	0,4	0,8	TEC1	√
orf19.4449	2,38E-04	2,7	1,5	2,1		
orf19.1822	3,82E-04	2,7	0,7	0,8	UME6	√
orf19.2457	2,60E-05	2,7	1,0	1,1		
orf19.176	1,34E-02	2,6	1,0	0,8	OPT4	
orf19.5015	1,35E-02	2,6	1,5	1,9	MYO2	√
orf19.921	3,46E-03	2,5	0,9	2,5		
orf19.5691	4,86E-03	2,5	1,3	1,9	CDC11	
orf19.4450	6,43E-06	2,5	1,3	1,9		
orf19.347	3,13E-05	2,5	1,2	2,2	RSN1	
orf19.467	9,55E-03	2,5	0,9	1,3		
orf19.1693	1,67E-02	2,5	1,3	1,5	CAS4	√
orf19.7531	3,81E-02	2,5	1,0	2,0		
orf19.7612	4,08E-04	2,5	1,4	2,3	CTM1	√
orf19.682	3,45E-04	2,5	1,5	2,1		
orf19.3642	4,86E-03	2,5	1,3	1,6	SUN41	
orf19.3438	1,58E-02	2,5	1,4	1,9		
orf19.4618	1,01E-02	2,5	1,5	2,1	FBA1	
orf19.4591	3,48E-03	2,5	9,0	8,7	CAT2	
orf19.395	1,44E-02	2,5	1,4	1,7	ENO1	
orf19.4109	8,25E-04	2,5	1,8	2,5	PMT4	√
orf19.4477	2,10E-02	2,5	2,1	4,8	CSH1	√
orf19.1690	3,75E-02	2,5	1,3	1,5	TOS1	
orf19.4590	5,52E-03	2,4	0,7	0,6	RFX2	√
orf19.5446	4,19E-02	2,4	0,8	0,9		
orf19.3070.1	8,32E-03	2,4	9,0	9,3		
orf19.4631	8,12E-05	2,4	0,6	1,7	ERG251	
orf19.1562	1,32E-02	2,4	1,4	1,3		
orf19.2333	9,82E-06	2,4	1,4	2,7		
orf19.7328	2,81E-04	2,4	1,8	2,7		
orf19.6573	1,90E-02	2,4	1,1	1,1	BEM2	√
orf19.5995	9,03E-04	2,4	1,2	1,9	MCA1	
orf19.2050	1,29E-02	2,4	1,4	2,0		
orf19.3448	2,05E-05	2,4	0,8	1,8		
orf19.730	5,60E-03	2,4	1,4	1,7		
orf19.1409.2	1,79E-05	2,4	0,6	0,7		
orf19.2803	1,95E-03	2,4	1,3	1,9	HEM13	

orf19.2626	1,17E-02	2,4	1,5	2,1	RGD2	
orf19.4998	6,57E-03	2,4	0,8	0,9		
orf19.999	2,58E-02	2,3	1,7	1,9	GCA2	
orf19.1401	4,17E-03	2,3	0,8	0,7	EAP1	
orf19.723	1,68E-02	2,3	1,1	1,3	BCR1	√
orf19.2699	2,38E-03	2,3	1,6	2,1	ABP1	√
orf19.3651	2,23E-03	2,3	2,0	2,4	PGK1	
orf19.2574	1,68E-02	2,3	1,5	1,9		
orf19.4975	2,34E-03	2,3	1,0	0,9	HYR1	√
orf19.5686	2,02E-02	2,3	1,7	2,4		
orf19.4035	2,21E-04	2,3	1,2	1,5	PGA4	
orf19.85	3,65E-03	2,3	0,5	0,5	GPX2	√
orf19.5495	3,11E-02	2,3	1,4	1,5		
orf19.4655	1,03E-04	2,3	1,2	1,2	OPT6	
orf19.6491	3,57E-02	2,3	5,9	6,1		
orf19.2157	8,23E-03	2,3	1,0	1,5	DAC1	√
orf19.6882	2,19E-02	2,3	1,0	2,6	OSM1	
orf19.374	3,80E-02	2,3	1,3	1,5		
orf19.1189	7,85E-04	2,3	1,1	1,8		
orf19.7654	2,53E-03	2,3	1,1	2,4	CPR6	
orf19.6176	4,01E-05	2,2	1,4	1,9	SEC61	√
orf19.2340	3,66E-02	2,2	1,5	2,3	CDC48	
orf19.1478	2,77E-02	2,2	1,6	2,0		
orf19.399	1,07E-02	2,2	1,5	1,7		
orf19.4936.1	2,04E-02	2,2	0,2	0,5		
orf19.473	4,53E-02	2,2	14,0	20,4	TPO4	
orf19.6327	6,62E-04	2,2	1,5	2,0	HET1	
orf19.5459	1,07E-02	2,2	1,4	1,5		
orf19.4735	1,25E-03	2,2	2,2	2,6		
orf19.13	6,00E-03	2,2	1,7	2,5		
orf19.5445	1,79E-02	2,2	1,1	1,6	GLO3	
orf19.7304	6,62E-04	2,2	0,6	1,1		
orf19.7297	5,54E-06	2,2	1,4	1,9		
orf19.2947	4,06E-02	2,2	1,0	1,0	SNZ1	√
orf19.2098	1,13E-02	2,2	1,8	1,9	ARO8	√
orf19.7308	5,39E-03	2,2	1,3	1,7	TUB1	
orf19.6842	7,72E-04	2,2	1,4	1,5		
orf19.1408	1,12E-04	2,2	1,5	1,9		
orf19.3038	1,34E-03	2,1	1,2	1,6	TPS2	

orf19.316	6,73E-04	2,1	1,7	2,4	SEC13	
orf19.1180	1,58E-04	2,1	1,5	1,9		
orf19.1254	4,37E-04	2,1	1,8	2,4	SEC23	√
orf19.1949	1,52E-02	2,1	1,5	1,8	VPS1	
orf19.3944	1,21E-02	2,1	1,3	1,6	GRR1	
orf19.3409	6,61E-03	2,1	1,2	1,8	SEC12	
orf19.3744	1,50E-03	2,1	1,4	1,6		
orf19.4082	1,28E-02	2,1	2,1	2,7	DDR48	
orf19.3969	3,57E-02	2,1	0,9	1,1		
orf19.7258	6,35E-03	2,1	1,0	1,5	DDI1	
orf19.3941	2,71E-02	2,1	1,3	1,0	URA7	
orf19.4183	3,44E-03	2,1	1,3	1,7	MUC1	
orf19.4451	3,73E-02	2,1	1,3	1,8	RIA1	
orf19.6763	7,99E-03	2,1	2,0	2,2	SLK19	
orf19.4433	1,11E-03	2,1	1,0	1,5	CPH1	
orf19.7242	5,98E-03	2,1	1,6	2,0	NCR1	
orf19.1191	4,02E-05	2,1	1,3	2,0		
orf19.3166	1,64E-02	2,1	1,4	1,8		
orf19.7053	3,04E-02	2,1	1,0	1,3	GAC1	
orf19.1760	3,40E-03	2,1	0,9	1,0	RAS1	
orf19.7538	1,99E-02	2,1	1,0	1,2		
orf19.6112	1,24E-02	2,0	0,8	1,4	CTA2	
orf19.4024	1,65E-05	2,0	1,5	1,9	RIB5	
orf19.734	1,50E-03	2,0	1,8	2,4	GLK1	
orf19.7600	1,87E-04	2,0	0,9	2,2	FDH3	
orf19.5823	1,36E-02	2,0	1,4	1,9	SGT2	
orf19.1964	1,45E-05	2,0	0,9	1,0		
orf19.92	8,93E-05	2,0	1,6	1,8		
orf19.6249	3,69E-02	2,0	1,1	1,5	HAK1	
orf19.979	3,49E-02	2,0	1,4	2,0	FAS1	
orf19.1120	2,37E-02	2,0	0,9	0,9	FAV2	√
orf19.5171	3,61E-02	2,0	1,6	2,1	PMT1	
orf19.4255	1,55E-03	2,0	2,2	2,6	ECM331	
orf19.3811	1,24E-02	2,0	1,6	1,9	GYP1	
orf19.1193	1,59E-02	2,0	1,0	1,8	GNP1	
orf19.6527	2,06E-02	2,0	1,3	1,6		
orf19.1979	4,12E-02	2,0	1,0	1,4	GIT1	
orf19.6165	3,10E-02	2,0	1,3	1,7	KGD1	
orf19.6155	3,15E-02	2,0	1,0	1,0		

orf19.4054	3,21E-04	2,0	0,9	1,5	CTA24	
orf19.7329	7,39E-04	2,0	1,2	1,7		
orf19.2296	3,39E-02	2,0	1,0	1,7		
orf19.3419	2,90E-02	2,0	0,4	0,6	MAE1	
orf19.1203	1,95E-04	2,0	1,6	1,9	SRO77	
orf19.6448	2,56E-02	2,0	1,1	1,4		
orf19.1871	2,31E-02	2,0	1,3	1,8		
orf19.69	3,02E-02	2,0	1,2	1,4		
orf19.5148	7,96E-03	2,0	1,4	1,5	CYR1	
orf19.281	1,78E-02	2,0	1,0	1,2		
orf19.5463	7,74E-03	2,0	1,1	1,3		
orf19.2163	2,58E-02	2,0	1,0	1,3		
orf19.1288	4,56E-03	2,0	4,1	7,5	FOX2	

Supplementary Table S4. The significantly differentially expressed genes were obtained by comparing "Experiment 37°C" with "Experiment CLA 37°C ". Differentially expressed genes were defined by a fold difference > 2 and a P-value <0.05

Systematic	P-value	Norm	Norm	Norm	FC ratio	Name
		37°C	30°C + CLA	37°C + CLA	37°C + CLA vs 37°C	150 genes
orf19.272	4,58E-04	1,8	16,3	16,9	9,3	FAA21
orf19.3442	1,02E-03	0,4	4,8	3,7	9,2	
orf19.473	1,59E-03	2,2	14,0	20,4	9,2	TPO4
orf19.3684	3,37E-03	1,4	12,4	12,4	9,0	
orf19.3040	4,79E-05	1,5	11,5	12,8	8,6	EHT1
orf19.1652	2,76E-03	1,8	14,4	11,8	6,5	POX1-3
orf19.7520	1,83E-03	1,6	12,1	10,2	6,4	POT1
orf19.344	9,81E-04	1,2	4,1	6,8	5,8	
orf19.3548.1	1,24E-03	0,8	3,9	4,4	5,7	WH11
orf19.1704	3,01E-04	1,1	10,9	5,9	5,3	FOX3
orf19.1655	1,78E-04	1,2	8,3	5,9	4,9	PXP2
orf19.1809	2,34E-03	1,9	8,0	8,4	4,4	
orf19.164	6,45E-05	1,0	3,0	4,1	4,3	
orf19.1709	5,05E-06	1,1	6,1	4,5	4,0	
orf19.5215	8,53E-04	1,9	5,9	7,4	4,0	TES15
orf19.4807	3,14E-04	0,9	3,2	3,3	3,9	
orf19.3070.1	1,54E-03	2,4	9,0	9,3	3,8	
orf19.23	1,01E-02	0,8	2,9	3,0	3,7	RTA3
orf19.6254	8,47E-05	1,1	4,7	3,8	3,6	ANT1
orf19.1027	1,12E-02	1,2	2,9	4,1	3,6	PDR16
orf19.4591	3,49E-03	2,5	9,0	8,7	3,5	CAT2
orf19.7111.1	2,72E-02	0,2	1,0	0,6	3,0	SOD3
orf19.6007	1,71E-03	0,6	2,2	1,9	3,0	
orf19.6229	1,55E-02	3,2	8,7	9,4	2,9	CAT1
orf19.7371	2,13E-02	0,9	1,8	2,6	2,9	
orf19.2531	9,92E-03	0,5	1,6	1,6	2,9	CSP37
orf19.5218	1,35E-03	1,0	4,2	2,9	2,8	

orf19.5217	7,12E-05	1,3	4,5	3,7	2,8	TES1
orf19.4211	1,85E-02	0,1	2,0	0,4	2,7	FET3
orf19.6838	2,70E-03	1,3	7,8	3,7	2,7	
orf19.1089	3,27E-03	0,3	1,4	0,9	2,7	PEX11
orf19.1473	1,54E-03	0,5	1,4	1,3	2,7	
orf19.2175	1,70E-02	1,6	2,0	4,3	2,7	
orf19.86	2,68E-03	1,4	3,7	3,8	2,7	
orf19.6491	1,65E-02	2,3	5,9	6,1	2,7	
orf19.4121	7,12E-03	1,6	5,7	4,3	2,7	
orf19.4122	1,87E-05	1,0	3,9	2,5	2,7	
orf19.6830	1,38E-03	0,9	2,5	2,3	2,6	
orf19.7513	2,22E-03	1,1	2,7	2,9	2,6	
orf19.5267	1,40E-03	0,4	1,0	1,1	2,5	
orf19.1890	1,73E-03	1,3	2,5	3,1	2,5	
orf19.7306	1,36E-03	0,9	1,6	2,3	2,4	
orf19.5753	7,51E-03	0,4	1,5	0,9	2,4	HGT10
orf19.909	7,13E-03	0,6	1,5	1,5	2,4	STP4
orf19.7569	1,76E-02	0,3	0,6	0,8	2,4	SIK1
orf19.54	1,73E-02	0,2	1,1	0,5	2,4	RHD1
orf19.6577	6,39E-03	1,0	2,3	2,5	2,4	FLU1
orf19.4763	2,68E-03	0,9	1,5	2,0	2,4	
orf19.5078	8,98E-03	0,6	1,4	1,4	2,4	
orf19.3133	3,47E-02	1,4	3,1	3,3	2,3	GUT2
orf19.5286	9,48E-03	1,2	1,8	2,7	2,3	YCP4
orf19.5193	7,16E-04	1,1	1,8	2,5	2,3	
orf19.2599	2,78E-03	1,3	3,1	2,9	2,3	
orf19.7288	2,88E-03	1,1	3,4	2,5	2,3	
orf19.4216	3,19E-02	1,4	2,4	3,1	2,3	
orf19.6627	4,74E-03	1,0	2,1	2,2	2,2	
orf19.6058	7,32E-03	0,9	1,4	1,9	2,2	GLO1
orf19.3488	1,22E-02	1,3	3,4	2,9	2,2	
orf19.5285	5,36E-04	1,2	1,9	2,6	2,2	PST3
orf19.3139	2,50E-03	1,1	2,2	2,4	2,2	
orf19.4157	1,76E-04	1,1	3,7	2,3	2,2	SPS20
orf19.4041	9,70E-03	1,4	2,3	3,1	2,2	PEX4
orf19.6445	3,21E-02	1,2	3,4	2,5	2,2	ECI1
orf19.163	1,02E-03	0,6	1,2	1,2	2,1	
orf19.7	7,29E-03	0,9	2,8	1,9	2,1	
orf19.5921	1,39E-03	1,2	3,5	2,5	2,1	

orf19.2881	7,21E-04	0,8	1,4	1,6	2,1	MNN4
orf19.3668	9,12E-03	0,3	1,5	0,7	2,0	HGT2
orf19.2883	6,63E-04	0,9	2,1	1,9	2,0	CSO99
orf19.926	2,22E-02	0,5	1,1	1,0	2,0	EXO1
orf19.1300	1,54E-02	0,4	0,8	0,9	2,0	
orf19.2726	2,22E-02	0,8	2,1	1,6	2,0	
orf19.3364	1,49E-02	1,0	1,0	2,0	2,0	
orf19.3710	4,84E-03	1,4	0,5	0,7	0,5	YHB5
orf19.2823	1,53E-02	1,7	0,9	0,8	0,5	RFG1
orf19.2061	1,49E-02	1,3	0,9	0,6	0,5	
orf19.3941	2,10E-02	2,1	1,3	1,0	0,5	URA7
orf19.7304	7,80E-03	2,2	0,6	1,1	0,5	
orf19.6155	2,71E-02	2,0	1,0	1,0	0,5	
orf19.2947	4,32E-02	2,2	1,0	1,0	0,5	SNZ1
orf19.1760	2,51E-03	2,1	0,9	1,0	0,5	RAS1
orf19.5741	2,13E-02	1,4	0,7	0,7	0,5	ALS1
orf19.2685	4,73E-02	2,9	0,8	1,4	0,5	PGA54
orf19.2721	1,99E-02	1,5	0,7	0,7	0,5	
orf19.1996	3,28E-02	1,6	0,5	0,7	0,5	CHA1
orf19.3641	2,03E-02	1,5	0,7	0,7	0,5	
orf19.5455	1,42E-04	1,1	0,6	0,5	0,5	
orf19.3823	4,27E-02	3,2	1,1	1,5	0,5	ZDS1
orf19.2179	2,79E-02	1,1	0,7	0,5	0,5	SIT1
orf19.5431	2,47E-02	1,2	0,7	0,5	0,4	
orf19.1120	1,46E-02	2,0	0,9	0,9	0,4	FAV2
orf19.3219	3,71E-03	1,7	0,6	0,7	0,4	
orf19.6573	1,93E-02	2,4	1,1	1,1	0,4	BEM2
orf19.3232	1,47E-02	1,4	0,9	0,6	0,4	
orf19.4716	4,77E-02	1,0	0,9	0,4	0,4	GDH3
orf19.1490	3,28E-03	3,4	1,1	1,4	0,4	MSB2
orf19.2457	7,00E-03	2,7	1,0	1,1	0,4	
orf19.5308	2,28E-02	0,6	0,3	0,3	0,4	
orf19.2170	2,86E-02	2,8	0,9	1,2	0,4	PHM7
orf19.5094	9,66E-03	1,5	0,8	0,6	0,4	BUL1
orf19.4682	4,65E-03	1,1	0,8	0,5	0,4	HGT17
orf19.6459	1,32E-02	0,9	0,5	0,4	0,4	DPP3
orf19.5447	1,46E-02	2,7	0,8	1,1	0,4	HGT19
orf19.4975	7,27E-03	2,3	1,0	0,9	0,4	HYR1
orf19.5170	3,89E-02	1,5	0,8	0,6	0,4	ENA21

orf19.4998	1,63E-03	2,4	0,8	0,9	0,4	
orf19.1995	1,23E-02	0,8	0,4	0,3	0,4	
orf19.1715	2,53E-04	3,4	1,2	1,3	0,4	IRO1
orf19.4749	9,50E-03	1,0	0,5	0,4	0,4	
orf19.84	3,35E-03	1,5	0,6	0,6	0,4	CAN3
orf19.2833	6,36E-03	3,3	0,8	1,2	0,4	PGA34
orf19.6556	5,09E-03	1,6	0,7	0,6	0,4	
orf19.1799	1,84E-03	3,1	1,1	1,2	0,4	
orf19.5446	2,17E-02	2,4	0,8	0,9	0,4	
orf19.4056	4,00E-02	2,8	0,8	1,0	0,4	GAT2
orf19.6705	2,14E-02	2,9	0,9	1,0	0,3	
orf19.3746	1,23E-02	3,2	1,0	1,1	0,3	IFC1
orf19.7610	5,30E-04	1,3	0,5	0,4	0,3	PTP3
orf19.1822	9,88E-04	2,7	0,7	0,8	0,3	UME6
orf19.5585	3,92E-03	5,0	1,4	1,6	0,3	SAP5
orf19.1401	6,12E-03	2,3	0,8	0,7	0,3	EAP1
orf19.3718	2,25E-03	3,1	0,9	0,9	0,3	
orf19.5908	1,50E-02	2,7	0,4	0,8	0,3	TEC1
orf19.3419	7,08E-03	2,0	0,4	0,6	0,3	MAE1
orf19.176	2,70E-03	2,6	1,0	0,8	0,3	OPT4
orf19.1409.2	2,83E-05	2,4	0,6	0,7	0,3	
orf19.3475	1,29E-04	1,5	0,4	0,4	0,3	
orf19.4590	1,07E-03	2,4	0,7	0,6	0,3	RFX2
orf19.4936.1	3,63E-03	2,2	0,2	0,5	0,2	
orf19.2292	2,66E-03	3,3	0,8	0,8	0,2	
orf19.6169	4,83E-03	3,4	0,6	0,8	0,2	
orf19.85	1,04E-03	2,3	0,5	0,5	0,2	GPX2
orf19.5307	1,45E-02	1,1	0,3	0,2	0,2	JEN2
orf19.4555	6,28E-03	1,7	0,5	0,4	0,2	ALS4
orf19.2121	8,25E-03	1,6	0,5	0,4	0,2	
orf19.1097	1,70E-02	1,7	0,5	0,4	0,2	ALS2
orf19.4900	2,25E-05	1,2	0,4	0,2	0,2	
orf19.815	4,80E-03	6,4	0,9	1,2	0,2	DCK1
orf19.2210	1,39E-03	8,2	1,1	1,5	0,2	
orf19.6028	2,00E-05	4,6	0,7	0,8	0,2	HGC1
orf19.3981	9,28E-06	3,5	0,7	0,6	0,2	MAL31
orf19.2355	9,55E-04	3,5	0,5	0,6	0,2	ALS10
orf19.1321	1,30E-03	7,8	0,4	0,9	0,1	HWP1
orf19.701	1,56E-04	5,6	0,6	0,6	0,1	CFL11

orf19.5760	4,95E-05	12,6	0,2	1,3	0,1	IHD1
orf19.3384	1,98E-03	5,7	0,3	0,5	0,1	
orf19.1816	1,45E-03	5,6	0,3	0,5	0,1	ALS3
orf19.1327	3,33E-04	21,4	0,3	1,1	0,0	RBT1
orf19.3374	3,07E-03	22,6	0,4	0,7	0,0	ECE1
orf19.2060	5,94E-04	10,9	0,2	0,2	0,0	SOD5

FORMATION ACADÉMIQUE

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PUBLICATIONS/COMMUNICATIONS

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- Characterization of pLJ42, a novel theta-replicating *Lactobacillus plantarum* plasmid. Communication orale prononcée dans le cadre de la Journée Perspective Bioprocédés, 14 septembre 2005, Institut de Recherche en Biotechnologie, Montréal.

- Characterization of pLJ42, a novel theta-replicating *Lactobacillus plantarum* plasmid. Affiche présentée au 8th Symposium on Lactic Acid Bacteria, 28 août-1er septembre 2005, Egmond aan Zee,Pays-Bas.